Exercise-Induced DNA Damage:
Effects of Hypoxia and Antioxidant Intervention

By

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Abstract

Reactive oxygen and nitrogen species (RONS) are continuously produced within the body during oxidative metabolism, and through normal immune function. When contained within a homeostatic balance, these molecules are part of a normal physiological function, and play integral signalling roles in cell growth, proliferation, and differentiation. This equilibrium is tightly regulated by a range of enzymatic and non-enzymatic antioxidants which seek to prevent detrimental reactions to biologically important macromolecules. The purpose of this thesis was twofold. Firstly, the use of various high-intensity exercise protocols was employed to investigate the effect on oxidative stress within circulating peripheral blood mononuclear cells and skeletal muscle tissue with a particular emphasis on DNA damage. Additionally, an environmental stressor was used via normobaric hypoxia to comprehensively elucidate the cell DNA damage-repair response, alongside systemic measures of oxidative stress following constant-load high-intensity exercise. Secondly, this thesis aimed to examine the efficacy of novel antioxidant supplementation on measures of exercise-induced oxidative stress; specifically, a plant-based combination of barley- and wheat-grass juice, and the mitochondrial-targeted antioxidant, Mitoquinone. Across all studies, it was confirmed that exercise of sufficient intensity (regardless of duration) provided a challenging physiological stress capable of inducing oxidative damage to DNA and lipids; likely through the generation of RONS, and concurrent reduction in antioxidant capacity. Furthermore, it was determined that peripheral blood mononuclear cells have an efficient and effective DNA damage-repair response, with maximal repair occurring within 24-hours for both single- and double-strand DNA damage; this response was marginally exacerbated in the hypoxic condition. The aforementioned modifications to DNA as a function of exercise, were concurrently aligned with systemic biomarkers of oxidative stress; including but not limited to, lipid peroxidation, lipid soluble antioxidants, and the ascorbyl free radical. With regards to plant-based antioxidant supplementation, although there was no meaningful prophylactic effect on a statistical level, a combination of barley- and wheat-grass juice did appear to provide a marginal protective effect against exercise-induced oxidative stress. On a more molecular level, mitochondrial-targeted supplementation for 21-days abrogated oxidative damage to the mitochondrial genome following high-intensity intermittent exercise; likely, through the scavenging of exercise-induced RONS, and/or

secondary oxidation products of mitochondrial lipid peroxidation. The results of the studies demonstrate an increase in oxidative stress following high-intensity exercise as evidenced by an increase in DNA damage (single-strand breaks, double-strand breaks, base oxidation) across nuclear and mitochondrial genomes, and changes to other systemic measures, such as: lipid hydroperoxides, lipid soluble antioxidants, and the ascorbyl free radical. The DNA damage-repair response was exacerbated by normobaric hypoxia with single- and double-strand DNA damage returning to baseline by 48-hours and 24-hours respectively. Finally, the data also indicates a potential prophylactic effect by supplementing with a plant-based nutraceutical, and mitochondrial-targeted antioxidant; however, further research is warranted to elucidate the underlying biochemical processes.

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Mr Josh Williamson

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Professor Gareth Davison

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Dr Ciara Hughes

Date……

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Table of Contents

Abbreviations

2,6-Diamino-4-Hydroxy-5-Formamidopyrimidine	FapyGua
3-Hydroxy-3-Methyl-Glutaryl-CoA	HMG-CoA
3-Hydroxy-3-Methyl-Glutaryl-CoA Reductase	HMGC
8-Oxo-2'-Deoxy-Guanine	8-oxo-G
Adenosine 5'-Triphosphate	ATP
Adenosine Monophosphate	AMP
Advanced Glycation End-Product	AGE
Alkoxyl Free Radical	RO·
Alpha Lipoic Acid	ALA
Ascorbyl Free Radical	Asc·
Ataxia Telangiectasia Rad3-Related/Mutated	ATR/ATM
Barley-Wheat Juice	BWJ
Base Excision Repair	BER
Breast Cancer Susceptibility Gene	BRCA
Calcium	Ca^{2+}
Catalase	CAT
Circulating Angiogenic Cells	CAC
Copper-Zinc	CuZn
Deoxyribonucleic Acid	DNA
Diatomic Oxygen	O_2
Diglyceride	DAG
Dihydrolipoic Acid	DHLA
Double Strand Break	DSB
Electron Spin/Paramagnetic Resonance	ESR/EPR
Electron Transport Chain	ETC
Endonuclease III	ENDO III
Exonuclease	EXO
Extracellular	EC
Farnesyl Diphosphate	FPP
Ferric Iron	Fe^{3+}
Ferrous Iron	Fe^{2+}
Flavin Adenine Dinucleotide	$FADH_2$
Formamidopyrimidine Glycosylase	FPG

Glutathione Peroxidase	GPx
Glutathione Reductase	GR
Glyceraldehyde 3-Phosphate Dehydrogenase	GAPDH
High Intensity Intermittent Exercise	HIIE
Homologous Recombination Repair	HRR
Hydrogen Peroxide	H_2O_2
Hydroxyl Free Radical	OH·
Hypochlorous Acid	HOCL
Lipid Hydroperoxide	LOOH
Lipid Soluble Antioxidants	LSA
Long-Amplicon Quantitative Polymerase Chain Reaction	LA-qPCR
Low-Density Lipoprotein	LDL
Malondialdehyde	MDA
Manganese	Mn
Messenger RNA	mRNA
Mismatch Repair	MMR
Mitochondrial DNA	mtDNA
Mitochondrial-Targeted Quinone	MitoQ
Mitochondrial-Targeted Vitamin E	MitoVitE
Monoamine Oxidase	MAO
Myeloperoxidase	MPO
N-Acetyl-Cysteine	NAC
Nicotinamide Adenine Dinucleotide Phosphate	NADPH
Nicotinamide Adenine Dinucleotide	NADH
Nitric Oxide Free Radical	NO·
Nitric Oxide Synthase	NOS
Non-Homologous End Joining	NHEJ
Nuclear Factor	NF
Nucleotide Excision Repair	NER
O^6-Methylguanine DNA Methyltransferase	MGMT
Oxidised Glutathione	GSSG
p53 Binding Protein 1	53BP1
Peripheral Blood Mononuclear Cells	PBMC
Peroxiredoxins	PRDX

Peroxyl Free Radical	$ROO^{\cdot}$
Peroxynitrite	$ONOO^{-}$
Peroxynitrous Acid	ONOOH
Phospholipase A_2	PLA_2
Polyunsaturated Fatty Acid	PUFA
Protein Kinase C	PKC
Reactive Oxygen and Nitrogen Species	RONS
Reduced Glutathione	GSH
Ribonucleic Acid	RNA
Semiquinone Radical	$Q^{\cdot -}$
Single Cell Gel Electrophoresis	SCGE
Singlet Oxygen	$^1O_2$
Superoxide Dismutase	SOD
Superoxide Free Radical	$O_2^{\cdot -}$
Thiobarbituric Acid Reacting Substances	TBARS
Thioredoxins	TRX
Total Antioxidant Capacity	TAC
Tricarboxylic Acid	TCA
Triphenylphosphonium	TPP+
Tumour Necrosis Factor	TNF
Uncoupling Protein	UCP
Water	H_2O
Xanthine Dehydrogenase	XDH
Xanthine Oxidase	XO

List of Figures

List of Tables

Scientific Communications Associated with this Thesis

Publications

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Index of Appendices

Chapter One

Chapter 1:
General Introduction

1.0 Introduction

Oxygen was first discovered in the early-to-mid 1770s, when the English chemist, Joseph Priestley described the capability of "*dephlogisticated air*" to increase the survival of mice within a sealed container, and additionally demonstrated the ability to reignite an ember of wood (Priestley, 1775). It is understood that Joseph Priestley's obsession with the theory of phlogiston's prevented him from furthering this monumental discovery. Although Priestley is often credited with this accomplishment, the first person to discover oxygen was Carl Wilhelm Scheele who, in 1772, isolated "*fire-air*" based on its ability to combust with mercuric oxide and potassium nitrate (Scheele, 1977). Finally, in 1778, Antoine Lavoisier was the first scientist to correctly quantify the combustible properties of the gas and provided a more descriptive definition; famously coining the gas, '*oxigene*'. Collectively, these three scientists conceptualised and provided the foundations for all photosynthetic and cellular respiratory research to come (West *et al.*, 2014).

Priestley forewarned that life could become enervated as a consequent of the gas' purity; however, it wasn't until the late 20th century were the adverse effects of oxygen was first documented. Paul Bert (Bert, 1943), and later James Lorrain Smith (Edmonds *et al.*, 1992), respectively highlighted central nervous system toxicity, and pulmonary toxicity as a result of hyperoxia; thus, the oxygen paradox was conceived. The chemical reactivity of oxygen hangs in the balance between our reliance on the oxidation of compounds to sustain human life (namely, carbohydrate and fat), and the toxic effects that are detrimental to human life.

The mechanism of action behind oxygens virulent effect was first elucidated in 1954, when Rebeca Gerschman and colleagues (1954) documented the radiation- and hyperoxia-induced generation of oxidising free radicals was comparable to that of oxygen toxicity. In the same year, Barry Commoner and colleagues (1954), published an equally compelling report, detailing the use of electron paramagnetic resonance to detect free radicals within living cells. These two papers arguably launched the area of free radical biology and medicine; solidified by the exponential rise in research publications.

Free radicals (e.g., superoxide, hydroxyl radical) are a group of short-lived, highly reactive biological molecules (Halliwell, 2006); their varying range of reactivity is paramount to their specific impact at the molecular level. Initially thought to be a harmful by-product of oxidative phosphorylation, free radicals have been implicated in a variety of physiological process within cells; such as, immune responses (oxidative burst), to signalling pathways (NF-κβ, MAPK) (Zhang *et al.,* 2016). The significance of free radicals in the progression of pathological diseases is now well documented (Alfadda & Sallam, 2012). The endogenous generation of free radicals, coupled with their removal by cellular antioxidant mechanisms, occurs continuously to prevent the potential damaging effects of free radicals to deoxyribose nucleic acid (DNA), ribonucleic acid (RNA), lipids, and proteins; collectively known as oxidative stress (Patel *et al.*, 2017).

With specific interest to DNA, it is documented that free radicals can interact with nucleic acids, resulting in structural modifications and potential mutagenic effects (Evans & Cooke, 2006). Specifically, the hydroxyl radical can perturb structures of all components of DNA inducing a plethora of DNA-specific lesions; such as single-strand breaks, double-strand breaks, and base damage. These lesions can have downstream mutagenic consequences, and as a result, directly affect DNA replication and transcription (Jena & Mishra, 2012). Given the potential pathophysiological relationship between DNA damage and free radicals, it is surprising that the majority of cellular components under normal circumstances are error free. For one, specialised enzymes protect DNA from erroneous and mutagenic effects by executing approximately 10^{16}-10^{18} repair events per cell per day. This requires the recognition of the specific DNA lesions, and the subsequent recruitment of the corresponding repair process; although the intricacies of these processes are not fully elucidated, base excision repair, nucleotide excision repair, mismatch repair, and double strand break repair are thought to be the primary DNA repair pathways (Cadet & Wagner, 2013). There are a myriad of exogenous and endogenous sources of free radicals with potential of inducing damage to DNA and other biomolecules, however, the primary focus of this thesis examines exercise-induced DNA damage and other markers of oxidative stress.

The relationship between exercise and oxidative stress was pioneered by Dillard *et al.* (1978), and Brady *et al.* (1979), who demonstrated an increase in expired pentane as a result of exercise; indicating that exercise causes lipid peroxidation. This increase in oxidative stress was later confirmed by Davies *et al.* (1982) using electron

paramagnetic resonance (EPR) spectroscopy in the skeletal muscle of rats. It is proposed the increased physiological demand of exercise causes an increase in mitochondrial electron transport chain activity, NADPH oxidase, xanthine oxidase, and phospholipase A_2-dependent processes (Powers & Jackson, 2008); in turn, increasing the likelihood of superoxide generation, and downstream hydroxyl radical formation. Due to the high reactivity and short half-lives of free radicals, EPR spectroscopy is the only methodology capable of direct quantification of free radical production. As a result, indirect measurement of free radicals focus on more stable molecular products of free radical reactions to quantify oxidative damage (Fisher-Wellman & Bloomer, 2009); these include, but not limited to nucleic acids [8-hydroxy-2-deoxyguanosine (8-OHdG), oxidized DNA bases and strand breaks, lipid hydroperoxides, and oxidised proteins. A summary of additional key milestones within the area of exercise-induced oxidative stress are highlighted in Table 1.1.

Table 1.1 Seminal papers associated with the development of the area of exercise and free radical research

Reference	**Key Finding**
Chance & Williams (1956)	Mitochondria produce reactive species
Harman (1956)	Free Radical Theory of Aging
McCord & Fridovich (1969a,b)	Discovery of superoxide dismutase
Dillard *et al.*, (1978)	Exercise-induced oxidative stress
Davies *et al.*, (1982)	Skeletal muscle produced reactive species
Quintanilha *et al.*, (1983a,b)	Exercise improves antioxidant capacity
Sies & Cadenas (1985)	Definition of oxidative stress
Novelli *et al.*, (1990); Shindoh *et al.*, (1990)	Reactive species contribute to muscle fatigue
Hammeren *et al.*, (1992)	Exercise training variables are associated with adaptations in primary antioxidant capacity
Reid *et al.*, (1993)	Redox status modulates force production

Notwithstanding the known effect of exercise-induced free radical production on important biomolecules such as DNA, the attenuation of free radicals via endogenous enzymatic and non-enzymatic antioxidants have also been used as a method to quantify oxidative stress. Additionally, the use of exogenous antioxidant supplementation (pharmacological or natural food sources) for the purpose of mitigating exercise-induced oxidative damage is equivocal. For one, research has shown that antioxidant supplementation can reduce (Ryan *et al.*, 2010), or indeed contribute to oxidative stress (Poljsak *et al.*, 2013). Moreover, others have proposed antioxidant supplementation may attenuate the beneficial adaptive responses associated with exercise (Gomes-Cabrera *et al.*, 2006). More recently, the efficacy of mitochondrial-targeted antioxidants has been examined within clinical populations (Orsucci *et al.*, 2019). To date, no research has investigated the effect of mitochondrial-targeted supplementation of exercise-induced oxidative stress.

1.1 Experimental Aims and Objectives

The primary aim of this thesis is to further develop the narrative aligned to high-intensity exercise and DNA damage. The comprised studies have employed the use of (i) maximal, exhaustive exercise, (ii) high-intensity steady state exercise, and (iii) high-intensity intermittent exercise. Although exercise was utilised as the primary mechanism to induce oxidative damage, severe hypoxia was also used as an independent oxidative stressor. A natural plant-based antioxidant comprising of barley grass and wheat grass was used to examine the efficacy of this novel nutraceutical on oxidative stress responses following exercise. Additionally, with the growing interest in mitochondrial dysfunction and the therapeutic use of targeted antioxidants, the efficacy in attenuating exercise-induced oxidative damage through the use of the mitochondrial-targeted antioxidant, Mitoquinone, was employed as a supplement intervention. Oxidative stress was investigated in a variety of biological sample types including lymphocytes, plasma, serum and mitochondrial DNA, extracted from peripheral mononuclear blood cells and skeletal muscle tissue.

In addition to the human work, the medium of cell culture work was incorporated to further elucidate the effect of high-intensity exercise on the DNA damage and/or repair response. Firstly, human primary lymphocytes were cultured to allow for the detection of residual and/or unrepaired DNA damage for up to 72-hours

following hypoxic exercise. Secondly, C2C12 mouse myoblasts were used as a positive control for mitochondrial DNA damage following hydrogen peroxide exposure.

The study aims are outlined below:

1.1.1 Study One

Aim: To investigate the potential prophylactic effects of a novel plant-based nutraceutical product on oxidative stress biomarkers (including DNA damage) as induced by incremental, exhaustive exercise.

1.1.2 Study Two

Primary Aim: To robustly examine changes in the DNA damage response and systemic oxidative stress following constant-load high-intensity exercise in hypoxia.

Secondary Aim: To investigate the DNA repair response following constant-load high-intensity exercise in hypoxia using human lymphocytes.

1.1.3 Study Three

Aim: To determine the efficacy of acute and chronic supplementation of the mitochondrial-targeted antioxidant, MitoQ, on mitochondrial genome DNA damage following high-intensity intermittent exercise.

Secondary Aim: To investigate the mitochondrial DNA damage response within C2C12 mouse myocytes following hydrogen peroxide exposure using long-amplicon PCR.

Chapter Two

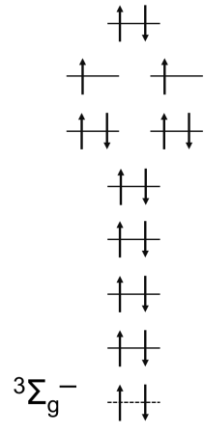
Chapter 2:
Review of Literature

2.0 Free Radical and Reactive Oxygen Species

A free radical, is defined as any species capable of independent existence that contains one or more unpaired electrons (Gutteridge & Halliwell, 1994). The first pervasive empiricism of a free radical was in 1900, when organic chemist Moses Gomberg published an epochal study on the discovery of the first 'stable' free radical; triphenylmethyl (Gomberg, 1900; Wang, 2010). Six years prior to the discovery of triphenylmethyl, in a letter Henry Fenton disclosed that in the presence of iron (II) and hydrogen peroxide, tartaric acid becomes oxidised (i.e. the gain of oxygen and/or loss of electrons) in a distinct manner, generating dihydroxymaleic acid (Fenton, 1894); however, the involvement of a free radical within this reaction was largely overlooked. Fenton and others, subsequently elucidated the damaging nature to organic matter from this reaction (Symons & Gutteridge, 1998); solidifying the central role of Fenton chemistry in modern free radical biology and medicine. Commoner and colleagues later in 1954 discovered the presence of free radicals within biological systems using electron paramagnetic resonance (Commoner *et al.*, 1954; Phaniendra *et al.*, 2015)

Free radicals are biologically reactive molecules (represented by a superscript dot) which are generated through a range of physiological and pathophysiological processes (Valdez *et al.*, 2000). Free radicals are implicated in the progression of a number of diseases including, but not limited to, type 2 diabetes mellitus (Bo *et al.*, 2013) and a range of cancers (Valko *et al.*, 2007; Trachootham *et al.*, 2009). According to the definition presented by Gutteridge and Halliwell (2015) diatomic (O_2), or ground state oxygen, is itself, a di-radical and ubiquitous in aerobic systems; these characteristics make it central to many biological processes. The molecular arrangement of ground state oxygen contains two free electrons occupying two molecular orbitals as represented by Figure 2.1.

Figure 2.1. Molecular arrangement of ground state oxygen.



These two free electrons have the same quantum spin state and thus cannot pair. In accordance to the Pauli Principle, ground state oxygen cannot accept a pair of electrons from a single molecular orbital (Gilbert, 1981). It would be logical to discern that having two unpaired electrons in two molecular orbitals would make ground state oxygen highly reactive; however, this is not the case. Due to the spin restrictions, oxygen prefers to gain electrons one at a time. This principle of reactivity applies to all free radicals and states the paramagnetic characteristics of the species is derived from the electron spin state and the magnitude of the magnetic moment (Halliwell & Gutteridge, 2007). The reaction of free radicals with other radicals, and indeed non-radicals, usually occur during oxidation and reduction reactions (i.e. the loss or gain of electrons, respectively). Four types of reactions involving free radicals within biological systems currently exist, (i) initiation reactions, (ii) propagation reactions where free radical numbers remain constant, (iii) propagation reactions where free radical numbers reduce, and (iv) termination reactions, which can be summarised within the following equations (Kehrer *et al.*, 2015):

i. $X – Y \rightarrow X^{\cdot} + Y^{\cdot}$

ii. $X – Y + X^{\cdot} \rightarrow X – X + Y^{\cdot}$

iii. $R^{\cdot} + O_2(^{\cdot}O=O^{\cdot}) \leftrightharpoons ROO^{\cdot}$

iv. $X^{\cdot} + X^{\cdot} \rightarrow X – X$

(Halliwell & Gutteridge, 2015)

Termination reactions, by which free radicals react with other free radicals, rarely occurs in biological organisms. For the most part, free radicals will usually react with non-radicals thus propagating a radical; in turn, creating a chain of events that can lead to the destruction of many biological macromolecules (Halliwell & Gutteridge, 2015).

During the tetravalent reduction of oxygen to water during normal respiration, no oxygen intermediates are formed. However, oxygen can accept 'escaped' electrons in alternate univalent reduction reactions that results in the formation of reactive oxygen species (ROS); the one-, two-, or three-reduction of oxygen, generate the partially reduced species superoxide, hydrogen peroxide, and the hydroxyl radical respectively (Powers *et al.*, 2011). Due to the nature of free radicals, these propagation reactions continue until stable molecules are generated; for example, water. Table 2.1 outlines key free radical species and reactive oxygen and nitrogen species (RONS).

Table 2.1. An overview of the chemical and biological aspects of reactive oxygen and nitrogen species

Reactive Species	**Chemical Formula**	**Half-life (seconds)**
Superoxide	$O_2^{\cdot -}$	$1 \times 10^{-5*}$
Hydrogen Peroxide	H_2O_2	Stable*
Hydroxyl	$OH^{\cdot}$	$1 \times 10^{-9*}$
Alkoxyl	$RO^{\cdot}$	$1 \times 10^{-6*}$
Ascorbyl	$Asc^{\cdot}$	Stable†
Peroxyl	$ROO^{\cdot}$	7††
Singlet Oxygen	$^1O_2$	1×10^{-6}
Hypochlorous Acid	HOCL	Stable
Nitric Oxide	$NO^{\cdot}$	$1\text{-}10^{\#\dagger\dagger}$
Peroxynitrite	$ONOO^-$	0.05^{-1*}
Semiquinone	$Q^{\cdot -}$	Days††

*Source: *Finaud et al., 2006; #Janzen, 1994 †Shyu et al., 2014; ††Kehrer et al., 2010*

2.0.1 - Superoxide

The existence of superoxide anions ($O_2^{\cdot -}$) was first predicted by Linus Pauling in a seminal paper titled, *"The Nature of The Chemical Bond"* in 1931, where he discussed the theory of quantum mechanics behind one- and three-electron bonds (Pauling, 1931; McCord & Edeas, 2005). However, it wasn't until his reflections in 1979 that he realised the possibility of superoxide possessing sufficient stability, existing with a three-electron bond plus a single bond (Pauling, 1979; McCord & Edeas, 2005). Since

its conception, superoxide has been exponentially cited within the literature, and is a key component of redox biology and free radical research. Superoxide is a primary free radical species produced through the monovalent reduction of ground state oxygen ($O_2 + e^- \rightarrow O_2^{\cdot -}$; Halliwell, 1995).

Unlike more reactive molecules, superoxide acts as a reductant ($E°' = +940$ mV) in the generation of secondary molecules, such as hydrogen peroxide (Powers & Jackson, 2008). Although superoxide is impermeable to lipid membranes and reacts rapidly, its toxicity lies in its ability to inactivate iron-suflur cluster containing enzymes; in turn, liberating free iron in the cell (Imlay, 2014). Consequently, this can generate the highly reactive hydroxyl radical through Fenton chemistry (Halliwell & Gutteridge, 2007). Superoxide is produced *in vivo* by physiological processes such as oxidative phosphorylation and oxidase-enzymes, however, exposure to radiation and ultraviolet light can also generate said radical (Heck *et al.*, 2003; Azzam *et al.*, 2012).

2.0.2 - Hydrogen Peroxide

By definition, hydrogen peroxide (H_2O_2) is not a free radical due to its full outer valence, however, it can be characterised as a ubiquitous oxidising agent; and thus, classified as a ROS (Jensen, 2003). The physicochemical properties of hydrogen peroxide, allows it to fulfil the criteria associated with signalling molecules (D.Autréaux & Toledano, 2007); for one, the kinetic and spatial relationships of a given RONS determine the specificity of its interaction with effectors within signalling pathways (Forman *et al.*, 2014).

The generation of hydrogen peroxide is driven through several monovalent and divalent reduction reactions (Breton-Romero & Lamas, 2014; Sies, 2017); including NADPH oxidases, xanthine oxido-reductase, uncoupled endothelial nitric oxide synthase and the electron transport chain (Lassegue *et al.*, 2012; Brand, 2016). Other sources of hydrogen peroxide production include lipoxygenase, cyclooxygenase and the cytochrome P450 system (Cai, 2005); however, over 30 enzymatic sources have been identified (as reviewed by Go *et al.*, 2015). The generation of hydrogen peroxide is formed primarily through the dismutation of superoxide:

$$2\,O_2^{\cdot -} + 2H+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

Due to a high activation energy requirement, hydrogen peroxide is relatively unreactive with most biological molecules, including low-molecular weight antioxidants; as a result, many of the reactions derived from hydrogen peroxide are kinetically driven (Winterbourn, 2013). Within the Fenton-mediated production of free radicals, a Haber-Weiss reaction can also occur (Suresh & Annam, 2013). These reactions are illustrated as below (Winterbourn, 2013):

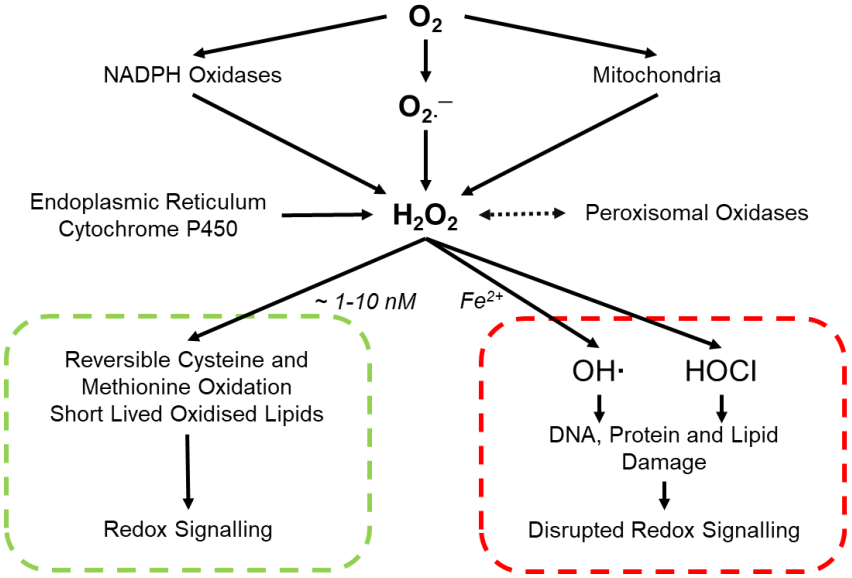
$$O_2\cdot^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH\cdot + OH^-$$

$$\textit{Net Reaction}: H_2O_2 + O_2\cdot^- \rightarrow OH\cdot + OH^- + O_2$$

As outlined by Sies (2017), cellular control of hydrogen peroxide is achieved through the fine balance of generation (Nauseef, 2014), translocation (Henzler & Steudle, 2000; Bienert *et al.*, 2007), and removal (Antunes *et al.*, 2002; Rhee & Woo, 2011). Cobley and colleagues (2015) postulate that hydrogen peroxide can act as an upstream bifurcation point between exercise-induced redox signalling and damage. For one, mitochondrial- and/or Fenton-mediated generation of hydrogen peroxide have the potential to damage nuclear and mitochondrial DNA (Murphy, 2012). It is equally plausible to infer that DNA damage can act as an indirect activator of redox-sensing pathways aligned to the regulation of DNA repair proteins (Radak *et al.*, 2013). On the contrary, detoxification of hydrogen peroxide via peroxiredoxins, glutathione peroxidases, and protein thiols, is likely to activate redox signalling pathways (Cobley *et al.*, 2015). In support of this concept, hydrogen peroxide is a known multifunctional, oxygen-derived metabolite which can exert both physiological and pathological effects associated with oxidative distress and oxidative eustress (Niki, 2016; Ursini *et al.*, 2016). This relationship is summarised in Figure 2.2 below.

Figure 2.2. The generation of hydrogen peroxide and downstream consequences. Hydrogen peroxide may act as a redox signal via reversible modifications as indicated within the green dashed lines. Similarly, when hydrogen peroxide accumulates in high concentrations, Fenton reactions may occur; in turn, producing the hydroxyl radical and downstream damage to DNA and other biomolecules.



Adapted from Sies (2017).

2.0.3 - Hydroxyl Radical

The hydroxyl radical (OH·), is the most damaging oxidant in biological systems (reduction potential E°' = +2310 mV), with no specificity in preference for co-reactants (Forman *et al.*, 2010). Additionally, the existent of the hydroxyl radical within biological systems can only be confirmed by circumstantial and indirect evidence (Pastor *et al.*, 2000; Powers & Jackson, 2008). Estimates of OH· accumulation indicate that individual cells can produce 4 million hydroxyl molecules per day (Ayala *et al.*, 2014); resulting in a wide range of molecular modifications (Kehrer *et al.*, 2010).

Within eukaryote cells, the primary mechanism of hydroxyl radical production is known to occur through Fenton chemistry as depicted above. Biological systems tightly regulate the redox status of cells, modulating free transition metals; namely iron (Valko *et al.*, 2007). The short half-life and high reactivity of the hydroxyl radical suggests all radical associated effects are within 50 nm from site of generation (Pryor, 1994); in turn, producing an unpredictable trend of molecular changes (Kehrer *et al.*, 2015).

2.0.4. Singlet Oxygen

Singlet oxygen ($^1O_2$) is a highly reactive radical derived from molecular oxygen when it enters an excited state; this can function as two entities (namely 1Δg and 1Σg),

achieved by overcoming the spin restriction associated with molecular oxygen (Agnez-Lima *et al.*, 2012). In contrast to molecular oxygen, singlet oxygen is highly reactive with electron-rich biological molecules such as lipids (Girotti, 1985; Frankel, 1998), thiols (Buettner & Hall, 1987; Rougee *et al.*, 1988), ascorbate (Bodannes & Chan, 1979; Rougee & Bensasson, 1986; Bisby *et al.*, 1999) and DNA (Cadet *et al.*, 2008; Agnez-Lima *et al.*, 2012).

With respect to the enzymatic generation of singlet oxygen, it is thought one potential mechanism occurs through the inflammatory process; specifically, from eosinophils, macrophages and neutrophils (Cilento & Nascimento, 1993; Teixeira *et al.*, 1999). Due to its reactive nature, and availability of biological targets, it has been suggested that singlet oxygen has a total life span of approximately 3 µs to around 100 ns (Moan & Berg, 1991; Schweitzer & Schmidt, 2003). It should be noted however, as outlined by Powers and colleagues (2011), the existence of singlet oxygen within skeletal muscle still remains equivocal.

2.0.5. Hypochlorite

Hypochlorite is a ROS produced through the enzyme myeloperoxidase (MPO); this typically occurs from the interaction of hydrogen peroxide in the presence of chloride ions with MPO (Weiss *et al.*, 1982). Although primarily involved within microbicidal immune defence, this ROS has accessory roles associated with the generation of the family of strong oxidising agents known as chloramines (Thomas *et al.*, 1986; Hammer *et al.*, 2001).

With respect to the biological production of hypochlorite *in vivo*, the likely source is during oxidative burst associated with neutrophils and monocytes (Hammer at al., 2001). This revelation is significant as there is evidence demonstrating hypochlorites ability to react with DNA and proteins (Hawkins & Davies, 1998; Hawkins & Davies; 2002); consequently, generating reactive nitrogen-centred radicals and fragmentation of protein backbones (Wakins *et al.*, 2002).

2.1 - Reactive Nitrogen Species

2.1.1 - Nitric Oxide

Nitric oxide (NO·) is defined as a free radical due to an unpaired electron on the antibonding $2\pi^*_\gamma$ orbital, however, it doesn't partake in initiation reactions. Nitric oxide synthase (NOS) and NADPH catalyses the reaction of the non-essential amino acid, L-arginine, to form NO· and L-citirulline (Darley-Usmar *et al.*, 1995). Within biological systems, NO· can exert both autocrine or pancrine effects, allowing for the regulation of various biological systems including the immune and cardiovascular systems (Pacher *et al.*, 2007). The primary function of NO· is to act as a mediator of vasodilation, however, it has also been implicated in inflammatory processes, pain perception, and platelet function (Mackenzie *et al.*, 2008). Similarly, to oxygen-centred radicals, the overproduction of nitrogen-based radicals can be characterised as nitrosative stress (Ridnour *et al.*, 2004), which has the potential to propagate nitrosylation reactions leading to altered protein architecture, and inhibition of their normal physiological function (Valko *et al.*, 2007). NO· is relatively unreactive to non-radicals, yet it will readily react with superoxide causing the generation of the potent oxidant, peroxynitrite (Kadlec *et al.*, 2016).

2.1.2 – Peroxynitrite

It was first thought that NO· acted as an antioxidant by scavenging superoxide (Feigl, 1988; McCall *et al.*, 1989); however, it was later discovered that the hydroxyl radical could be produced more efficiently from peroxynitrite compared to the Fenton reaction or the Haber-Weiss reaction (Beckman *et al.*, 1990; Durley-Usmar *et al.*, 1992; Hogg *et al.*, 1992). Peroxynitrite can be categorised as a highly toxic, reactive nitrogen intermediate which is produced by the reaction of superoxide with NO· (McCafferty, 2000). This reaction occurs at a rate constant of 7×10^9 M^{-1} s^{-1} and can be demonstrated below:

$$O_2^{\cdot -} + NO^{\cdot} \rightarrow ONOO^-$$

The rate of production of peroxynitrite can be as high as 50-100 μM per min (Alvarez *et al.*, 2004). In addition to peroxynitrites ability to cross cellular membranes, it can oxidise sulfhydryls, iron-sulfur centres and zinc-thiolates (Radi *et al.*, 1991a; Castro *et al.*, 1994; Crow *et al.*, 1995). Furthermore, peroxynitrite has been shown to

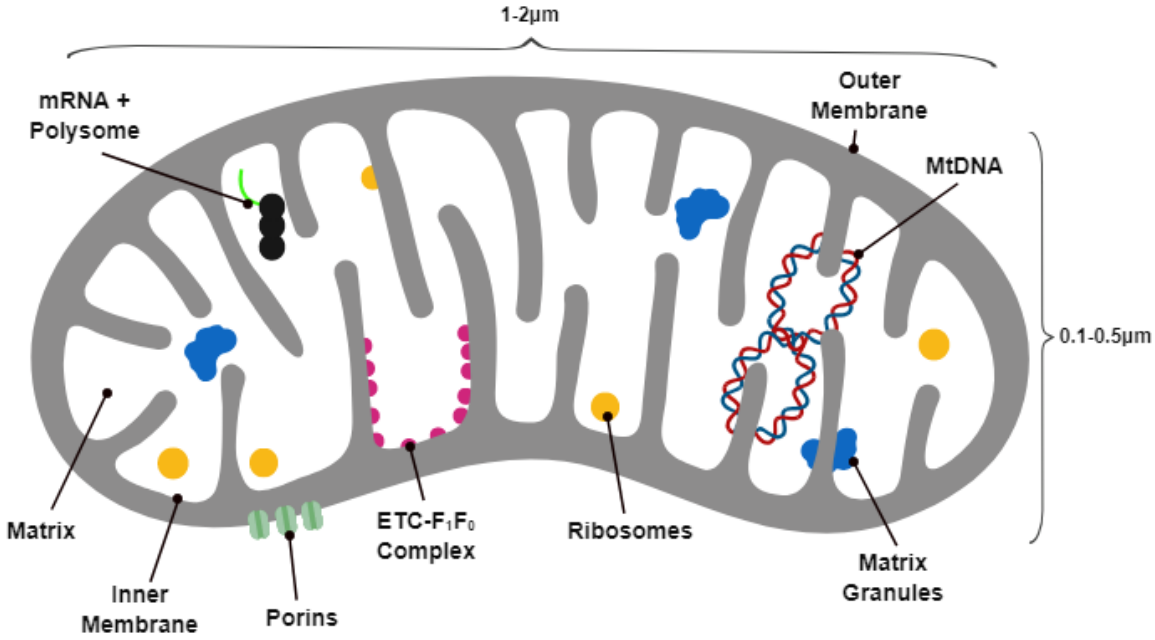
initiate lipid peroxidation (Radi *et al.*, 1991b), and has been suggested to play a critical role in inflammatory diseases (Pacher *et al.* 2007).

2.2 – In Vivo Formation of RONS

2.2.1 – Mitochondrion

Mitochondria are central cellular organelles within energy metabolism and generate ATP through the citric acid cycle, coupled with the electron transport chain (Bertram *et al.*, 2006). More recently, there has been a growing appreciation for other physiological functions of mitochondria such as apoptosis (Yee *et al.*, 2014), cell cycle progression regulation (Antico-Arciuch *et al.*, 2012), and the production of RONS (Hekimi *et al.*, 2016). An overview of the key components of the mitochondria is depicted in Figure 2.3.

Figure 2.3. Structural overview of the mitochondria and key organelle components.



The large integral proteins of the electron transport change differ greatly with regards structure and function. Complex I (NADH:ubiquinone oxidoreductase), is the largest multi-subunit component of the electron transport chain which transfers electrons from nicotinamide adenine dinucleotide (NADH) to ubiquinone which helps contribute to the proton gradient with a stoichiometry of 4 $H^+/2e^-$ (Lenaz *et al.*, 2006). Mitochondrial complex II (succinate:ubiquinone oxidoreductase), is often thought of as

a separate entity and is comprised of four protein subunits which are encoded by the nuclear genome (Bliek *et al.*, 2017). In contrast to the involvement of NADH in Complex I, Complex II uses flavin adenine dinucleotide ($FADH_2$) to transfer electrons, however unlike other complexes, it does not contribute to the electrochemical, proton gradient. Complex III (cytochrome bc1), is comprised of 11 subunits controlled by both the mitochondrial and nuclear genome. Its primary function is to modulate the Q-cycle, as detailed by Crofts *et al.* (2008), whereby Coenzyme Q is oxidised, while simultaneously, cytochrome c becomes reduced; in effect, this process pumps protons into the intermembrane space. The final enzyme, Complex IV (cytochrome c oxidase) is the terminal oxidase comprising of 13 subunits and functions by transferring electrons from ferro-cytochrome c to molecular oxygen; consequently, contributing to the transmembrane electrochemical gradient (Rak *et al.*, 2016). In turn, this process drives the production of adenosine 5'-triphosphate (ATP) via the tetravalent reduction of oxygen to water (Davies, 1995) as shown below.

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$$

Mitochondrial redox research was progressed by Chance and colleagues in the early 1970's (Boveris *et al.*, 1972; Boveris & Chance, 1973); however, the work by Jensen in 1966, inferred a small percentage of oxygen contributed to the formation of hydrogen peroxide following NADH/succinate oxidation. The two major forms of ROS within the mitochondria are superoxide and hydrogen peroxide; specifically, it is thought that superoxide accumulates at a concentration of 5- to 10-fold higher in the matrix compared to the cytoplasm or nucleus (Cadenas, 2000; Brand, 2010). Mitochondrial ROS production can be viewed in Table 2.2.

Table 2.2. Chemical reactions associated with free radicals specific to the mitochondria.

Mitochondrial ROS Production	Chemical Reaction
Monovalent Reduction	$O_2 + e^- \rightarrow O_2^{\cdot -}$
Ferric Iron Oxidation	$O_2^{\cdot -} + Fe3+ \rightarrow Fe2+ + O_2$
Cytochrome C Oxidation	$Cyt\ c\text{-}Fe3+ + O_2^{\cdot -} \rightarrow cyt\ c\text{-}Fe2+ + O_2$
Dismutation	$2O_2^{\cdot -} + 2H+ \rightarrow H_2O_2$
Glutathione Peroxidase	$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$
Peroxiredoxins	$2H_2O_2 \rightarrow 2H_2O + O_2$
Fenton Chemistry	$Fe2+ + H_2O_2 \rightarrow Fe3+ + OH\cdot + OH-$

There are seven well-accepted sites of superoxide generation within mammalian mitochondria which include, pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, the FMN-containing NADH binding site of complex I, the ubiquinone reduction site of complex I, the electron transferring flavoprotein ubiquinone oxidoreductase, glycerol 3-phosphate dehydrogenase, and the outer quinone-binding site of the Q-cycle in complex III; the majority of which, produced at the iron-sulphur clusters and/or the flavin of complex I and site IIIQo of complex III (Brand, 2010).

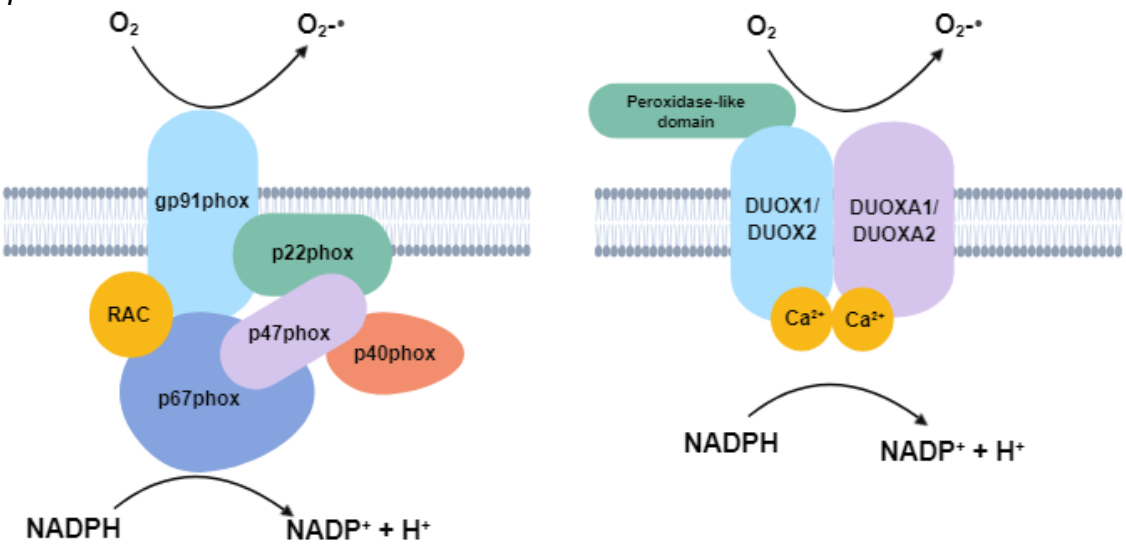
Early calculations postulated that 2-5% of total oxygen through the electron transport chain leads to superoxide formation (Boveris & Chance, 1973; Loschen *et al.*, 1974). It was thought that the increased contractile activity during exercise, and subsequent oxygen flux during mitochondrial respiration, cause arbitrary electrons to 'leak' from complex I (site IQ) and complex III (site IIIQo) (Brand, 2010); thus, causing an increase in superoxide production (Kanter, 1995; Urso & Clarkson, 2003). However, these assumptions are now known to be incorrect as these estimations are based on the use of antimycin to produce superoxide from complex III (Chance *et al.*, 1979). More recent observations by Saint-Pierre and colleagues (2002) demonstrate the production of superoxide at different sites of the electron transport chain in a variety of tissues including skeletal muscle, and suggest a conservative estimate of the total mitochondrial electron flow, of which approximately 0.15% contributes to superoxide anion formation; this coincides with earlier estimations by Hansford *et al.* (1997). In support of these suppositions, it is evident that during mitochondrial respiration is in state 4 which then transitions to active state 3 when muscle contraction begins; this is

classically characterised with a rapid breakdown in ATP and an accumulation of mitochondrial ADP (He *et al.,* 2016). Surprisingly, the rate of superoxide production is greater in basal state 4 respiration as opposed to active state 3 in skeletal muscle tissue; suggesting that during exercise, the mitochondria may not be the primary source of RONS (Kavazis *et al.*, 2009; Sakellariou *et al*., 2014).

2.2.2 – NADPH Oxidase

Nicotinamide adenine dinucleotide phosphate-oxidase, or NADPH oxidase (NOX), describes the transmembrane flavocytochrome complexes which facilitate the transportation of electrons across biological membranes (Bedard & Krause, 2007). Comprising from a group of seven enzymes, the NOX family produces superoxide through the univalent reduction of oxygen; contributing to oxidative stress, and redox homeostasis (Manea & Simionescu, 2012). Although the NOX/DUOX enzymes are structurally very similar, in that, they each share helices which allow them to bind to plasma membranes and have a binding C-terminal domain (Rada & Leto, 2008); they do possess minor structural alterations which allow for differentiation between the isoforms (Jackson *et al.*, 2010). The key structural components of the NOX/DUOX complexes can be observed in Figure 2.4.

Figure 2.4. Key components of the NADPH-oxidase complexes and the generation of superoxide.



Adapted from Rada & Leto (2008).

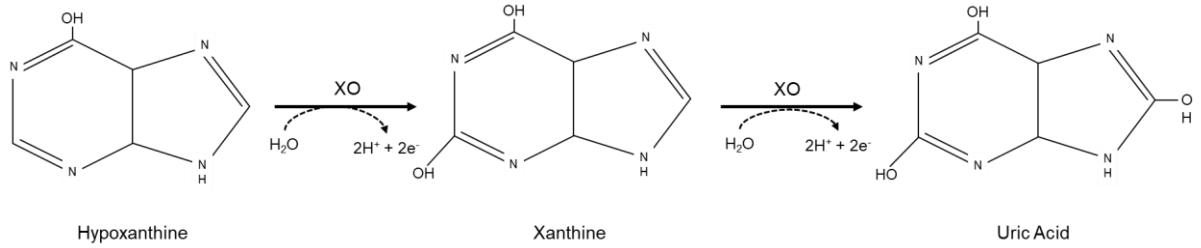
Depending on the cellular distribution, NADPH oxidase abstracts electrons from the cytosolic NADPH, across the plasma membrane, and into the phagosome, mitochondria, extracellular space, or other cellular compartments whereby oxygen acts as the electron acceptor (Lassegue & Griendling, 2010). This compartmentalisation of the NOX family allows them to possess a number of physiological roles, such as cellular differentiation, apoptosis, hormone release and calcium release, and more isoform-specific roles, such as host defence (NOX2; Rada *et al.*, 2008), otoconium formation (NOX3; Krause, 2004), and thyroid hormone iodination (DUOX2; Milenkovic *et al.*, 2007) to name a few. In addition to the physiological roles of NOX isoforms, a variety of pathological conditions have been associated with NOX-dysfunction including, chronic granulomatous disease (NOX2; Berendes *et al.*, 1957), gastrointestinal inflammation (NOX1/NOX2; Rokutan *et al.*, 2008), atherosclerosis (NOX5; Guzik *et al.*, 2008), and cystic fibrosis (DUOX1/2; Pongnimitprasert *et al.*, 2008).

More recently, research has shifted to focus on the interplay of NOX-compartmentalisation, the ability of cells to have multiple isoforms of NOX, and context-specific signalling pathways affected by RONS production (Ghouleh *et al.*, 2011). For instance, research demonstrates that the NADPH oxidase-mediated production of superoxide, modifies the ryanodine receptors within skeletal muscle tissue, and thus contributes to the release of calcium from the sarcoplasmic reticulum (Sun *et al.,* 2011). This release of calcium is essential for many physiological processes including cardiac and skeletal muscle contraction (Santulli & Marks, 2015). Due to the comprehensive cellular distribution, the generation of RONS via NADPH-oxidase has been implicated in the activation of various signalling pathways relating to regulation of the vascular system (Prieto-Bermejo & Hernandez-Hernandez, 2017), neuronal differentiation and signalling (Sorce & Krause, 2009), and the cellular stress response (Jiang *et al.*, 2011).

2.2.3 – Xanthine Oxidase

Xanthine oxidase (and xanthine dehydrogenase) is an enzyme that catalyses the oxidation of hypoxanthine to xanthine and subsequently, xanthine to uric acid (Enroth *et al.*, 2000). Specifically, xanthine oxidase acts as a reducing agent causing molecular oxygen to accept an electron; this results in the formation of the superoxide anion (Reznick *et al.*, 1998). This process is shown in Figure 2.5.

Figure 2.5. The enzymatic conversion of hypoxanthine to uric acid by the enzyme, xanthine oxidase. Unlike xanthine dehydrogenase, xanthine oxidase uses oxygen as an electron acceptor in the conversion of hypoxanthine to uric acid; consequently producing superoxide.



Purine degradation is catalysed by the enzyme xanthine oxidoreductase whereby electrons are transferred to a FAD moiety, consequently reducing NAD+ to NADH (Keeley *et al.*, 2010). It has been suggested that the breakdown of ATP to AMP via the adenylate kinase reaction during ischaemia may enhance the production of xanthine (McCord, 1985). Within biological systems, this ubiquitous metalloflavoprotein (~ 300kDa) exists as xanthine dehydrogenase and xanthine oxidase. Although xanthine dehydrogenase is the preferential isoform, it can be readily converted to xanthine oxidase following thiol oxidation of sulfhydryl residues or via proteolytic cleavage (Pacher *et al.*, 2006). In doing so, oxygen becomes the terminal acceptor; thus, yielding superoxide.

During aerobic exercise, ATP is broken down to allow for muscle contraction; however, in some instances AMP is formed owing to the accumulation of hypoxanthine, xanthine, uric acid, and indeed, superoxide; thereby exacerbating oxidative stress via xanthine oxidase (Mastaloudis *et al.*, 2001). Additionally, as a result of the energy demand associated with high intensity exercise, blood flow can be redirected to the working muscles, while simultaneously shunting blood flow from extraneous organs and tissues (Sarelius & Pohl, 2011; Joyner & Casey, 2015). This exercise-mediated ischemia-like effect, triggers the conversion of xanthine dehydrogenase to xanthine oxidase; consequently, upon exercise cessation and reoxygenation of shunted tissues, superoxide and hydrogen peroxide are generated. (Li & Jackson, 2002; Milar *et al.*, 2007). It has been hypothesised that this interconversion may be due to the contractile activity of exercise triggering a disturbance in calcium equilibrium, thereby allowing the activation of calcium dependent proteases (Vollaard *et al.*, 2005). It should be highlighted that these data have been reported mainly in animal models (Vina *et al.*, 2000; Gomez-Cabrera *et al.*,

2005); however, following strenuous eccentric exercise Hellsten and colleagues (1997) observed an increase in xanthine oxidase possibly due to secondary inflammatory processes, such as activated neutrophils.

2.2.4 – Autoxidation Reactions

Biological molecules such as ascorbic acid and glucose can slowly oxidise over time in the presence of oxygen; consequently, resulting in the formation of the superoxide free radical (Millar *et al.,* 1990). Superoxide produced from these reactions can potentiate further oxidation of the initiating molecule leading to a propagation reaction of free radical products (Halliwell, 1996; Young and Woodside, 2001). Furthermore, the autoxidation of oxyhaemoglobin to methaemoglobin in human erythrocytes can also produce superoxide (Rifkind *et al.*, 2013; Simoni *et al.*, 2009) and at any given time, it is thought that the concentration of methaemoglobin is 0.5-1% in normal healthy erythrocytes (Salvati & Tentori, 1981).

Although the transport of oxygen is tightly controlled due to the binding capacity of oxygen to Fe^{2+}, evidence by Rifkind and colleagues (2003) would suggest 3% of haemoglobin is autoxidised over a 24-hour period; this process is then exacerbated by several orders of magnitude when the partial pressure of oxygen reduces (Abugo & Rifkind, 1994). Due to the low cellular concentration of antioxidants within erythrocytes, the RONS generated from this process can result in membrane damage, or indeed, RONS leakage from the red blood cells (Nagababu & Rifkind, 2000; Nagababu *et al.*, 2002).

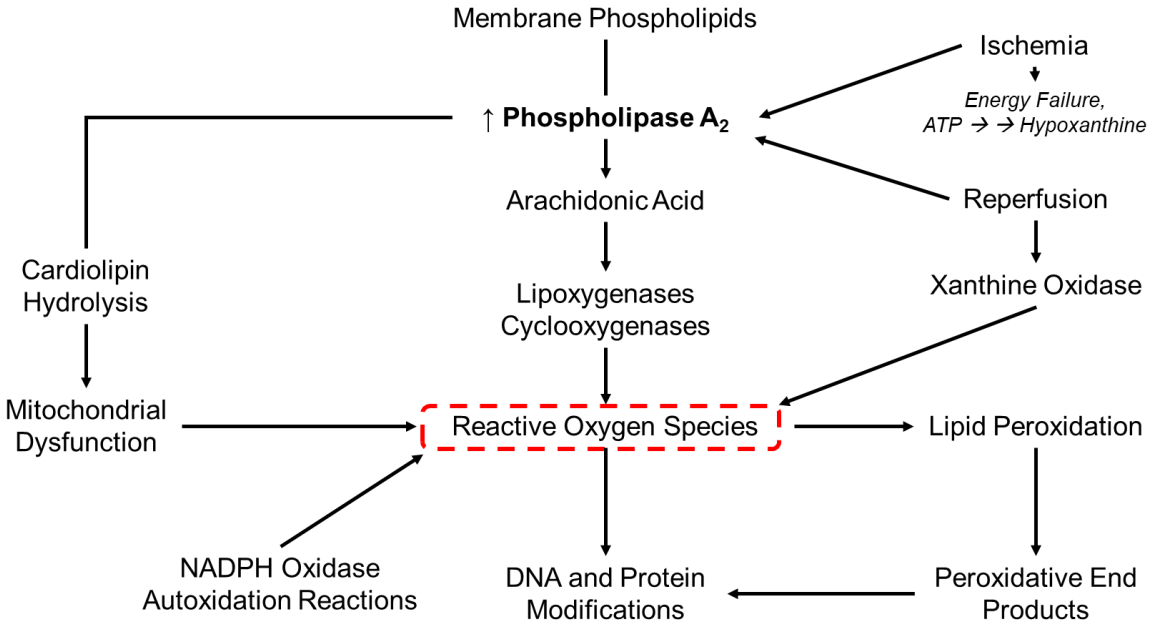
It is also important to highlight the role of glucose autoxidation within the production of RONS. It is known that blood glucose is tightly regulated below 5.6 mmol/L in a supply-and-demand manner; this of course achieved by the rate of glucose synthesis from the liver (Klover & Mooney, 2004) and kidney (via renal gluconeogenesis; Gerich *et al.*, 2001), and the uptake of glucose by tissues. Subsequently, glucose is oxidised with electrons being stored in NADH for the purpose of oxygen reduction during oxidative phosphorylation. During periods of excessive NADH accumulation, complex I of the electron transport chain will attempt to oxidise the increasing amounts of NADH. Conversely, this increased NADH to NAD+ ratio, causes a discourse in the amount of NAD+ molecules available for electron transport; thus, leaving for a greater propensity for electron leakage and univalent reduction of oxygen to superoxide (Ceriello, 2005; Hirst *et al.*, 2008; Kim *et al.*, 2008). It is also

noteworthy, the superoxide generated can consequently inhibit glyceraldehyde 3-phosphate dehydrogenase due to its redox-sensitive cysteine residue (Trentham, 1968; Rivera-Nieves *et al.*, 1999). As a result, the superoxide-mediated inhibition of glyceraldehyde 3-phosphate dehydrogenase and downstream effects of other glucose metabolism intermediates, reduces the efficiency of glycolysis and the Krebs cycle (Ussher *et al.*, 2012). Conclusively, these glycolytic intermediates need to be disposed off through additional processes namely, the polyol pathway, the hexosamine pathway, protein kinase C activation, advanced glycation end products, and the glyceraldehyde autoxidation pathway; all of which produce RONS as by-products. Although these mechanisms are minor and relatively insignificant in comparison to the other sources of RONS generation, these five pathways have been linked to the production of RONS and oxidative stress (Poitout & Robertson, 2002; Robertson, 2004; Davidson *et al.*, 2005; Adbul-Ghani & Defronzo, 2008; Poitout & Robertson, 2008).

2.2.5 –Phospholipase A_2 Processes

Phospholipase A_2 (PLA_2) is a family of enzymes which cleaves the peroxidised lipid resulting in the release of arachidonic acid (Zuo *et al.*, 2004). The family of PLA_2 enzymes can be categorised as Ca^{2+}-dependent cytoplasmic, and Ca^{2+}-independent cytoplasmic isoforms (Korbecki *et al.*, 2013). Both the dependent and independent form of phospholipase A_2 have shown to increase the generation of superoxide in biological systems (Nethery *et al.*, 2000; Powers *et al.*, 2011) by activating NADPH oxidase (Zhao *et al.*, 2002). As calcium is essential for contractile activity, it has been suggested that calcium-dependent phospholipase A_2 is a prominent source of superoxide within mitochondria and during exercise. Whereas, the calcium-independent phospholipase A_2 enzymes have been suggested to enhance cystolic RONS during resting conditions (Gong *et al.*, 2006; Powers *et al.*, 2011). Sources of RONS and the contribution of phospholipase A2 processes are summarised in Figure 2.6.

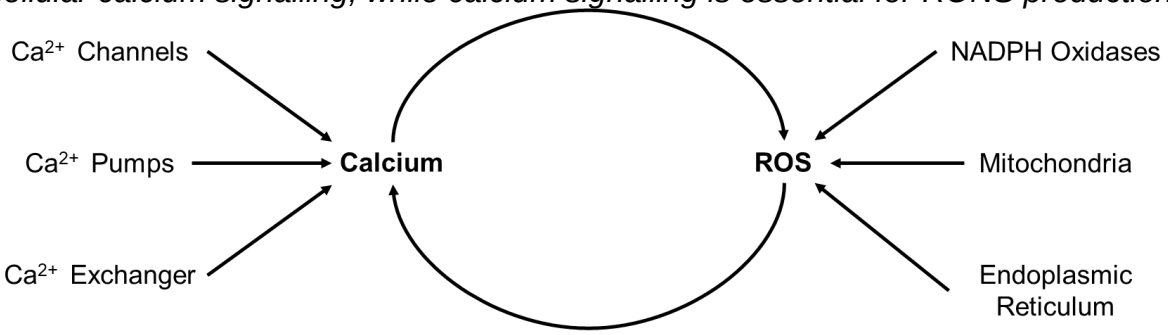
Figure 2.6. Contribution of phospholipase A2 to the generation of reactive oxygen species. The involvement of phospholipase A2 in RONS formation are produced by a number of cellular oxidative metabolic processes involving xanthine oxidase, NADPH oxidases, metabolism of arachidonic acid by cyclooxygenases and lipoxygenases, monoamine oxidases, and mitochondrial electron transport chain. Additionally, non-enzymatic sources are also evident such as autoxidation of catecholamines.



2.2.6 – Calcium Accumulation

Calcium (Ca^{2+}) is an important physiological element for signal transduction which has the ability to cross cell membranes through transmembrane calcium channels. The main role of calcium within mitochondria is the regulation of ATP synthesis (Adam-Vizi & Starkov, 2010). Berridge (2012) also states that calcium regulates apoptosis, cell metabolism, and gene expression. Additionally, Gorlach and colleagues (2015), identified the mutual interplay between calcium and other signalling molecules such as hydrogen peroxide (Holmstrom & Finkel, 2014) as illustrated in Figure 2.7.

Figure 2.7. The interplay between calcium and reactive oxygen and nitrogen species. Interactions between calcium and RONS are bidirectional wherein RONS can regulate cellular calcium signalling, while calcium signalling is essential for RONS production.



Within mitochondria, Krebs cycle enzymes and oxidative phosphorylation are stimulated by calcium, and as a result, the increased metabolic activity may consume more oxygen, potentially increasing RONS through electron leakage (Yoon *et al.*, 2004, Sohal & Allen, 1995). Mitochondrial metabolic state contributes to the interplay between calcium and RONS (Gorlach *et al.*, 2015). As such, during periods of low ATP synthesis, calcium uptake results in low RONS generation, and conversely, when the membrane potential of the mitochondrial is depolarised, RONS production is increased (Adam-Vizi & Starkov, 2010). There is also evidence to suggest that excess Ca^{2+} accumulation can alter the opening of mitochondrial permeability transitional pores, thus increasing the production of RONS (Yoon *et al.*, 2004). Although this mechanism is debateable, Castilho *et al.* (1995) and Crompton (1999), found that alterations to the mitochondrial membrane via excess calcium, can inhibit antioxidant scavenging ability and cause proteins to be released from the inner membrane of the mitochondria (Yan *et al.*, 2006).

2.2.7 – Peroxisomes

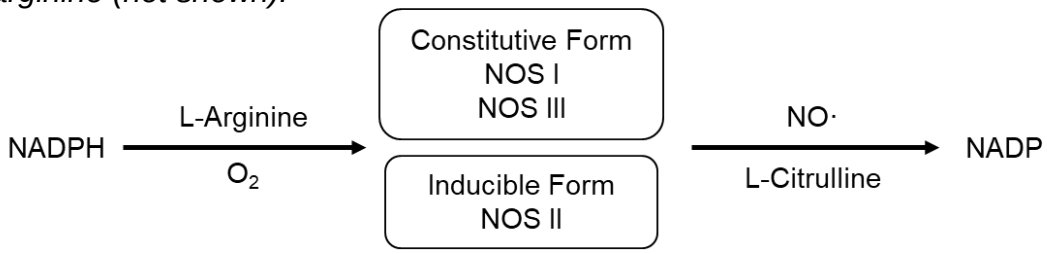
Peroxisomes are small, membrane-bound, self-replicating organelles which contain a number of enzymes including glycollate oxidase, urate oxidase and flavoprotein dehydrogenases (Alberts *et al.*, 2002). De Duve and Baudhuin (1966) were the first to discover that although peroxisomes are primary sources of hydrogen peroxide, they also contain significant concentrations of catalase. Peroxisomes contain no DNA or ribosomes, and thus import all their proteins from the cytosol. (Halliwell & Gutteridge, 2015). The primary function of peroxisomes is the oxidation of organic molecules; resulting in the formation of hydrogen peroxide (Wanders & Waterham., 2006).

Peroxisomes generate hydrogen peroxide through various metabolic processes including fatty acid α- and β-oxidation, enzymatic reactions of flavin oxidases, and the disproportionation of superoxide radicals. Many of the enzymes involved within these pathways generate free radicals as part of their normal catalytic function; for example, acyl-CoA oxidase, D-amino oxidase, xanthine oxidase, and inducible nitric oxide synthase (Fransen *et al.*, 2012). The primary source of hydrogen peroxide within mammalian peroxisomes is attributed to these oxidases that transfer hydrogen from their corresponding substrates, to molecular oxygen (Schrader & Fahimi, 2006).

2.2.8 – Nitric Oxide Synthase

Nitric oxide is synthesised from the substrate, L-arginine via the enzyme nitric oxide synthase (NOS) as outlined in Figure 2.8.

Figure 2.8. The nitric oxide pathway. NO· is a free radical that arises from the enzymatic oxidation of the amino acid L-arginine. NOS I is mainly present in the nervous system where it is responsible for the physiological release of NO· as a neurotransmitter. In the cardiovascular system, the most relevant isoenzyme for physiological purposes is NOS III. NOS II can be induced within smooth muscle cells, endothelial cells, and cardiac cells. It should be noted L-citrulline can be resynthesised to L-arginine (not shown).

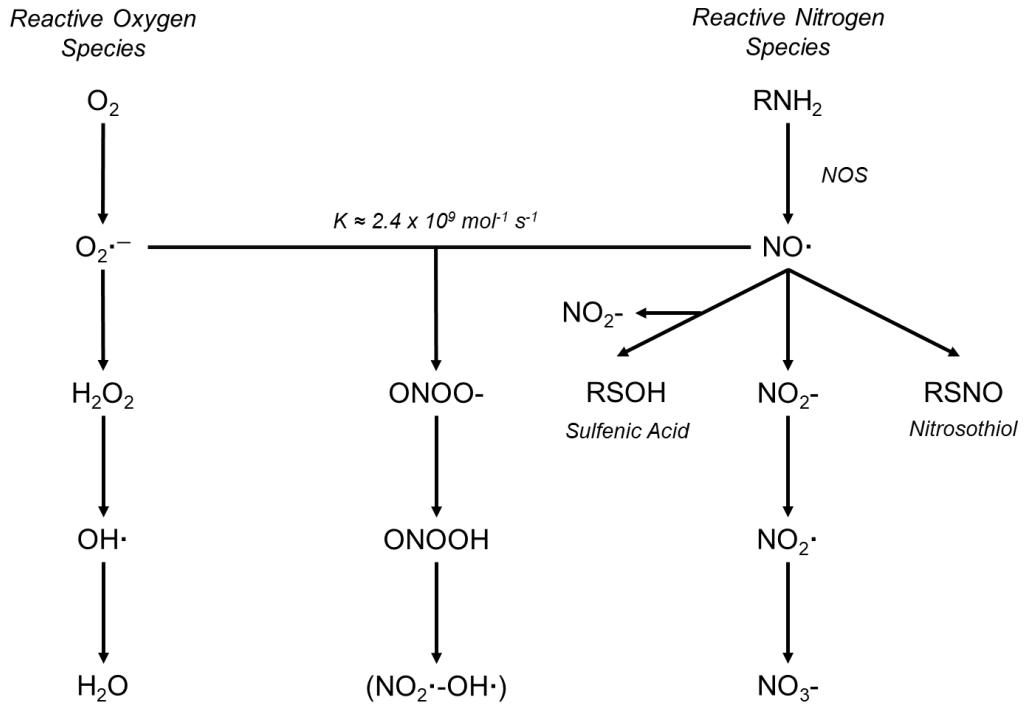


Typically characterised as a messenger/signalling molecule, NO· regulates a variety of biological functions including, neurotransmission (O'Dell *et al.*, 1991), vascular tone (Sandoo *et al.*, 2010), mRNA translation (Liu *et al.*, 2002), and ADP ribosylation-driven post-translational modifications (Brune *et al.*, 1994). NO· can be generated from one of three NOS-isoforms, each with their own specific attributes; neuronal (NOS I), inducible (NOS II), and endothelial (NOS III) (Forestermann, 2000). The activity of these isoforms is regulated at the transcriptional, translational, and post-translational levels (Stamler & Meissner, 2001).

Elucidating to the mechanism of action, NO· readily interacts with superoxide molecules to generate peroxynitrite (Figure 2.9); although itself not a free radical, it

does possess powerful oxidant capabilities (rate constant 7 x 10^9 M^{-1} S^{-1}), which can alter biological structures and deplete thiol groups (Uppu *et al.*, 1996; Pfeiffer *et al.*, 1999). The availability of NO· relies on the enzymatic and non-enzymatic generation, and removal of NO·, as a result of interactions between NO· and superoxide (Dyakova *et al.*, 2015). As superoxide accumulates, there is potential for a concomitant decrease in NO bioavailability, and increase in oxidative damage to cellular structures. In addition, there are data demonstrating the ability of exercise to increase the bioavailability of NO, improve endogenous antioxidant capacity, and reduce the expression of RONS-forming enzymes (Gliemann *et al.*, 2014).

Figure 2.9. The interplay between superoxide and nitric oxide, and the downstream propagation of other reactive oxygen-nitrogen species: parallel but connecting paths. Nitroxyl anion (NO^-), a one-electron reduction product of nitric oxide (NO·), is unlikely to arise from ·NO under physiological conditions, but is considered be a primary and more toxic product of NOS. Reaction of reactive nitrogen species with cysteine sulfhydryls can lead either to S-nitrosylation or to oxidation to the sulfenic acid, as well as to disulfide bond formation (not shown), all of which are potentially reversible. Peroxynitrite anion ($OONO^-$) and peroxynitrous acid (OONOH) have distinct patterns of reactivity. OONOH spontaneously decomposes via species resembling the reactive radicals, hydroxyl (OH·) and/or nitrogen dioxide (·NO_2).



Although these consequences are unwarranted, NO can play beneficial, protective roles by sequestering other radicals; the ability of NO to quench peroxyl radicals (k_2 = 1-3 x 10^{-9} dm^3 mol^{-1} s^{-1}), results in the inhibition of the lipid peroxidative process (Hughes, 2008). This has been supported across multiple cell types including endothelial cells and macrophage cells (Struck *et al.*, 1995; Hogg *et al.*, 1995). Additionally, within the context of terminating lipid peroxidation, NO has been shown to exert a greater antioxidant effect in comparison to α-tocopherol (O'Donnell *et al.*, 1997).

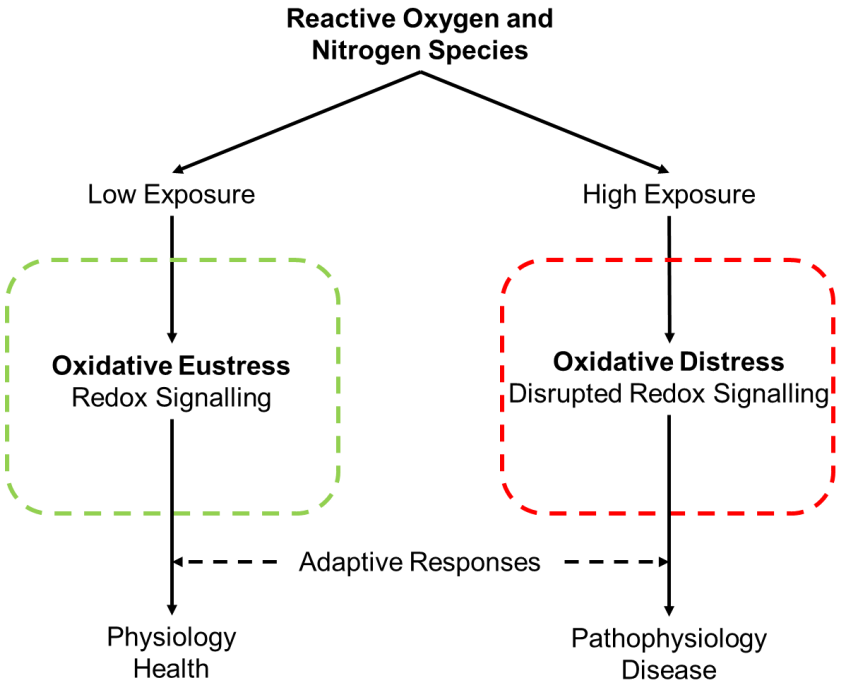
2.3 – Redox Biology, Oxidative Stress and Related Terminology

One of the major goals in biology in this new century is to completely understand the chemistry, biochemistry, and all aspects of the regulatory pathways of cells, tissues, and organisms. This initially began with the Human Genome Project in 1990 to understand the nature and functioning of genes and their corresponding proteins; more recently however, the focus has shifted to examining our knowledge and understanding of the *redoxome*. Defined by Buettner and colleagues in 2013, the redoxome is a composite of quantitative information on the redox enzymes and proteins as well as the unstable, quasi stable, and redox active species that determine the redox environment of cells and tissues. These unstable, quasi stable, and redox active species are fundamental regulators of genes, proteins, and connecting pathways and networks. It is now recognized by the greater research community that redox active molecules in conjunction with RONS are at the base of the regulation of biological processes (Foreman *et al*., 2003). These very reactive species set the redox state of the many redox couples and pathways in cells and tissues thereby leading to health or disease (Schafer & Buettner, 2001). Missing in all of the above is quantitative information on these transient, unstable, and quasi-stable redox active species that provide the connection between metabolism, protein function, lipid use and function, and gene expression. This is initiated with the use of dioxygen by cells (Wagner, 2011) and subsequent formation of reactive oxygen and nitrogen species, the concentration and redox state of the many thiols and disulfides (cysteine, glutathione, thioredoxin, glutaredoxin, the peroxiredoxins, mixed protein thiols *etc.*) as well as the actual concentrations of antioxidant enzymes (superoxide dismutase, glutathione peroxidases, peroxiredoxins, catalase, *etc*.). These enzymes have traditionally been considered solely as antioxidant enzymes; however, their cellular role reaches much further. Because they control the steady-state levels of RONS, they are involved in the regulation of signalling processes. In the broadest sense, redox biology aims at understanding the coupling and coordination between electron gradients and the overall organization of living organisms. In between, at the interface between electron flow and cellular functions, are the redox modifications of cellular components. As such, redox biology is an extremely broad field that covers metabolism, free radical and oxidative stress biology (Chance et al., 1979; Sies, 1986), sulfur and selenium chemistry (Jacob et al., 2003; Winterbourn & Hampton, 2008), and many other aspects. It is now known that physiological levels of intracellular and extracellular

RONS function as ‘triggers’ for signalling cascades whereas excessive accumulation of RONS is known to induce deleterious effects, causing oxidative damage to different kinds of biomolecules; these processes are referred to as oxidative stress.

Although not strictly defined, oxidative stress has been a concept in redox biology since the 1950s as work largely focussed on the correlation and mechanisms associated with oxygen toxicity and aging (Gerschman *et al.*, 1954; Harman, 1956). The first notable definition of the term ‘oxidative stress’ was proposed by Helmut Sies in 1985, describing oxidative stress as a relatively simple homeostatic equilibrium of antioxidants versus pro-oxidants (Sies, 1985; Sies & Cadenas, 1985). Later, Sies and Jones proposed a more inclusive definition taking into consideration oxidative damage to macromolecules and/or redox signalling (Sies & Jones, 2007); this relationship can be observed in Figure 2.10.

Figure 2.10. Relationship between oxidative stress and redox signalling. Low oxidant exposure allows for addressing specific targets in the use for redox signalling (oxidative eustress), whereas high exposure leads to disrupted redox signalling and/or damage to biomolecules (oxidative distress). Adaptive responses modulate and counteract. The outcome contributes to health and disease processes



Adapted from Sies (2018).

The model proposed by Sies (2018; Figure 2.10) centres around the idea that oxidative stress can be classified according to intensity, with the continuum ranging from physiological oxidative stress whereby redox signals address specific targets (Eustress), to toxic oxidative stress which damages unspecified biomolecules (Distress). This observation can be classically defined as the hormesis theory as developed by Stebbing (1982), and extended to various fields by others (Minois, 2001; LeBourg, 2003; Radak *et al.,* 2005). The term 'hormesis' refers to the beneficial effects of low exposure to potentially harmful substances; consequently, forming a bell-shaped curve. The basis of this hypothesis within the area of free radial biology is supported by the notion that RONS are physiological products of metabolism; whereby, at low concentrations have a stimulating effect (signalling, receptor stimulation, enzymatic stimulation etc.), while high concentrations can cause macromolecule damage and apoptosis (Radak *et al.,* 2005).

With specific attention to this thesis, it is well-established that exercise "releases" a set of local and systemic stressors that trigger integrated acute responses, which in the long-term result in phenotypic adaptations in all human body systems (Margaritelis *et al.,* 2020). Despite the traditional view that reactive species are exclusively detrimental molecules, recent evidence suggests that exercise-induced RONS are essential upstream signals for the activation of redox-sensitive transcription factors and the induction of gene expression associated with exercise. These redox processes are an integral part of the exercise-associated metabolism; the acute responses and training induced adaptations will be discussed later in section 2.5.6 of this chapter.

It should be noted, modifications to redox signalling can occur in the absence of oxidative damage, and vice-verse (Cobley *et al.*, 2015). More recently, Cobley and colleagues (2017) have presented recommendations when reporting on oxidative stress in order to prevent confusion and indeed interpretational errors. Firstly, oxidative stress must be strictly defined, and described in relation to the assay used. Secondly, oxidative stress must be interpreted impartially, unless evidence of functionality is presented; these recommendations also apply to nitrosative and reductive stress.

Powers and Jackson (2008) classified biomarkers which characterised *in vivo* oxidative stress into four main groups: (i) production of oxidants, (ii) concentration of antioxidants, (iii) products of oxidation, and (iv) redox balance. Due to the short half-life of many salient free radicals, direct measurement of oxidant production is virtually

impossible in living cells. However, by using techniques such as fluorescent probes and spin trapping (i.e. 5,5-dimethyl-1-pyrroline-N-oxide and N-*tert*-butyl-α-phenylnitrone), quantification of specific radical by-products can yield an accurate measure of oxidant production (Han *et al.*, 2000; Hwang & Kim, 2007). With that being said, oxidative stress encompasses redox balance, thus quantification of oxidants alone is not enough to accurately assess oxidative stress. With regards using antioxidant concentration as a measure of oxidative stress, antioxidant status (concentration or activity) can increase or decrease as a function of oxidative stress and vary depending on tissue type (Kawamura & Muraoka, 2018). Although they can be useful biomarkers, it should be highlighted that nutritional status of the participants may influence outcome measurements (Nordberg & Arner, 2001; El Abed *et al.*, 2011; Birben *et al.*, 2012). The third category of oxidative biomarkers relates to the products of oxidation to macromolecules. Biomarkers of lipid, protein and DNA oxidation are commonly used to quantify oxidative stress (Kasai, 1997; Hwang & Kim, 2007). Finally, redox markers such as the reduced/oxidised glutathione ratio, are extremely useful but can be influenced by improper sampling technique or handling of the tissue (Cobley *et al.,* 2017).

With all of these categories, a common issue which can affect sensitivity, and indeed accuracy, is the handling and processing of samples. As outlined by Halliwell & Gutteridge (2007), improper handling of samples can cause autoxidation resulting in the diminution of antioxidants from the sample. It is clear from the literature that there are a number of limitations with each biological measure of oxidative stress, and there is no optimal biomarker for the quantification of oxidative stress (Powers *et al.*, 2010). Therefore, it is widely advised that multiple biomarkers be used to provide a more comprehensive representation of oxidative stress *in vivo* (Powers & Jackson, 2008).

Similar to the original definition of oxidative stress as clarified by Sies in 1985, nitrative stress describes the physiological state whereby reactive nitrogen species (RNS) exceed the endogenous detoxification pathways associated with RNS. One of the most common sources of nitration within biological systems is through the action of peroxynitrite (Szabo *et al.*, 2007; Radi, 2013); this can be characterised through the addition of a nitro group ($-NO_2$) to a target compound. Nitration by peroxynitrite can interact with lipid peroxidation (Radi *et al.*, 1999), DNA adduct formation and strand breakage (Beckman *et al.*, 1990; Kennedy *et al.*, 1997), thiol oxidation (Quijano *et al.*, 1997; Bonini & Augusto, 2001) and tyrosine residues (Radi, 2004).

The final related term refers to the nitrosylation of redox active protein thiols and metallocofactors; this addition of a nitroso group (-NO) is known as nitrosative stress. This state of nitrosative stress is evidently involved in several signalling pathways and regulatory roles (Jaffrey *et al.*, 2001; Foster *et al.*, 2003; Liu *et al.*, 2004); with growing interest in the nitrosylation modification of post-translational proteins (Stamler *et al.*, 2001; Lane *et al.*, 2001; Foster *et al.*, 2003). Although the body of literature is clear regarding the physiological roles of nitrosative stress in biological systems, there is evidence directly implicating nitrosative stress in the pathology of several diseases (Dalle-Donne *et al.*, 2003; Klandorf & Dyke, 2012; Pandey *et al.*, 2013).

2.4 – Antioxidants

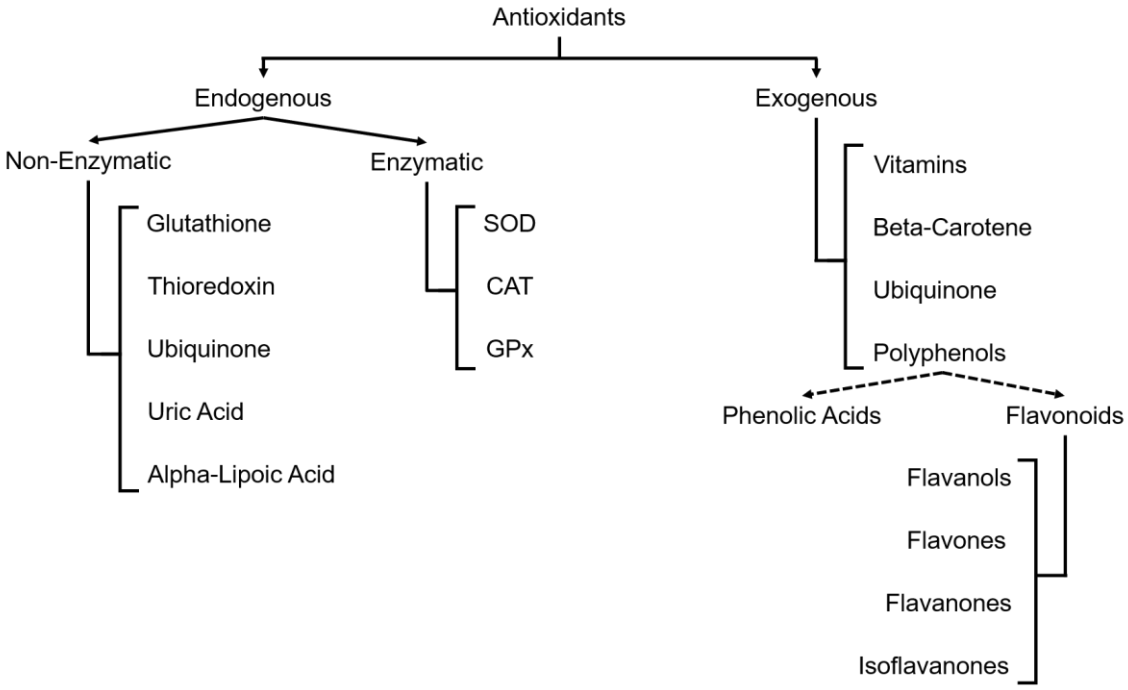
As previously established, oxygen is toxic to many aerobes which has resulted in the evolutionary development of a protective defence system comprising of enzymatic and non-enzymatic antioxidants. One of the first empirical reports on the relationship between free radical-induced toxicity and the prophylactic effect of antioxidants was published by Recknagel (1967), who proposed RONS-driven lipid peroxidation could be prevented by α-tocopherol supplementation.

Table 2.3. The general antioxidant attributes found within biological cells and their corresponding definitions. Adapted from Marambe & Wanasundara (2012).

General Antioxidant Principles	Definition
Reactive Species Removal	Certain antioxidants catalytically remove reactive species for example superoxide dismutase, catalase, and glutathione peroxidase
Controlling Reactive Species Formation	Molecules within this category comprises of proteins that minimise pro-oxidant availability such as iron or copper ions.
Protectors	These molecules minimise or prevent oxidative damage by other mechanisms. For example, enzymatic determination of mitochondrial PUFA or proteins that coat DNA.
Sacrificial	These are molecules which sacrifice themselves in place of more biologically important molecules. These include α-tocopherol, ascorbate, and urate.

Halliwell and Gutteridge (2015) define an antioxidant as any molecule capable of delaying, preventing, or removing oxidative damage to a target molecule; an overview of these functions is detailed in Table 2.3. This biological network of enzymatic and non-enzymatic antioxidants maintains RONS at a physiological threshold; thereby, enabling RONS to exert essential signalling processes (Barbieri & Sestili, 2012). An overview of the major antioxidants *in vivo* is depicted in Figure 2.11.

Figure 2.11. Overview of the major endogenous and exogenous antioxidants.



2.4.1 Enzymatic Antioxidants

2.4.1.1 Superoxide Dismutase

First discovered in 1969 by McCord & Fridovich, superoxide dismutase (SOD) is a primary antioxidant for the defence against superoxide anions and can be categorised as a ubiquitous metalloenzyme (Poljsak & Milisav, 2013). SOD exists as three isoforms within mammalian tissue (Zelko *et al.*, 2002); the molecular properties of these isoenzymes are summarised in Table 2.4. SOD catalyses the dismutation of superoxide, in turn producing hydrogen peroxide, which is summarised below:

$$2\,O_2^{\cdot-} + 2H+ \xrightarrow{SOD} H_2O_2 + O_2$$

Table 2.4. Summary of the molecular characteristics associated with SOD isoenzymes. Abbreviations – SOD – superoxide dismutase; Mt – mitochondria. Data based on the evidence reported in Hearn et al. (1999) and Suzuki et al. (2000).

	Location	Metal/ Monomer	Molecular Weight (kDa)	Subunit	Rate Constant with $O_2^{\cdot}$
SOD1	Cytosol and Mt intermembrane space	1 Cu, 1 Zn	32.5	Dimer	0.62×10^9
SOD2	Mt matrix	1 Mn	24.7	Tetramer	1.2×10^9
SOD3	Extracellular	1 Cu, 1 Zn	30	Tetramer	0.72×10^9

Superoxide radicals are relatively unreactive and do not react well with biological structures; although there are exceptions such as modifying the redox state of proteins, or the reduction of heme within cytochrome c (Weidinger & Kozlov, 2015). It is thought that SOD was an evolutionary adaptation for the survival of biological organisms; this is evident across multiple species (Elchuri *et al.*, 2005; Unlu & Koc, 2007), whereby deletion of one or more of the SOD enzymes leads to an increase in mortality. This has been confirmed in humans exhibiting spinal neuron apoptosis as a result of mutated SOD1 (Fridovich, 1995). Additionally, it could be conceivable that this fundamental necessity of SOD is reflected by its compartmentalisation across several subcellular locations; for instance, SOD2 exists to quench superoxide radicals produced within the mitochondria at a rapid rate ($\kappa \approx 2 \times 10^9$ M^{-1} s^{-1}) (Mailloux *et al.*, 2013).

The activity of these isoforms within skeletal muscle is not constant; research suggests that the activity of SOD can be altered as a function of exercise. The consensus of the literature demonstrates that exercise increases the activity of SOD1 and SOD2 ([20-112%], Criswell *et al.*, 1993; Oh-ishi *et al.*, 1997; Vincent *et al.*, 2000; Lawler *et al.*, 2006). With that being said, some data demonstrates that even after chronic endurance training, SOD activity remains unchanged (Laughlin *et al.*, 1990; Helsten *et al.*, 1996; Lambertucci *et al.*, 2007). It should be noted that there is little consistency with regards to methodology and training programs used; more so, it has been suggested by Oyanagui (1984) that there could be a 10-fold difference in variance in assays used to quantify SOD activity. This would potentially elude to the failure to

detect small differences of SOD activity due to the low sensitivity of assay. Furthermore, the physiological differences in animal and human tissue, and fibre type of the participants should not be ignored as increases of exercise-induced SOD activity is greater in type I and type IIa fibres (Powers *et al.*, 1994).

2.4.1.2 Catalase

Catalase is a monofunctional, tetrameric, haem-containing enzyme, found in almost all aerobically respiring organisms (Chelikani *et al.*, 2004) with the highest accumulation present in mature erythrocytes and the liver. The primary role of catalase is to generate oxygen and water from the oxidoreductase-decomposition of hydrogen peroxide (~10^7 M/Sec; Goyal & Basak, 2010) with iron or manganese acting as co-factors. Briefly, catalase acts through two mechanisms of action; the catalatic pathway ($2H_2O_2 \rightarrow O_2 + 2H_2O$) and the peroxidatic pathway ($H_2O_2 + AH_2 \rightarrow A + 2H_2O$) (Kabel, 2014).

As a result of the location within erythrocytes, catalase acts to protect haemoglobin, however it has also been implicated within inflammation, mutagenesis, and prevention of apoptosis; which appears to be dependent on the type of tissue and the mechanism of oxidant-mediated tissue injury (Ho *et al.*, 2004). To this end, catalase, in addition to SOD and glutathione peroxidase, are considered first line defence antioxidants. This is evident in individuals with catalase polymorphisms or acatalasemia, who tend to have increased susceptibility to certain pathological conditions such as type 2 diabetes mellitus, cancer, vitiligo, and Alzheimer's disease (Zamocky & Koller, 1999; Goth *et al.*, 2004).

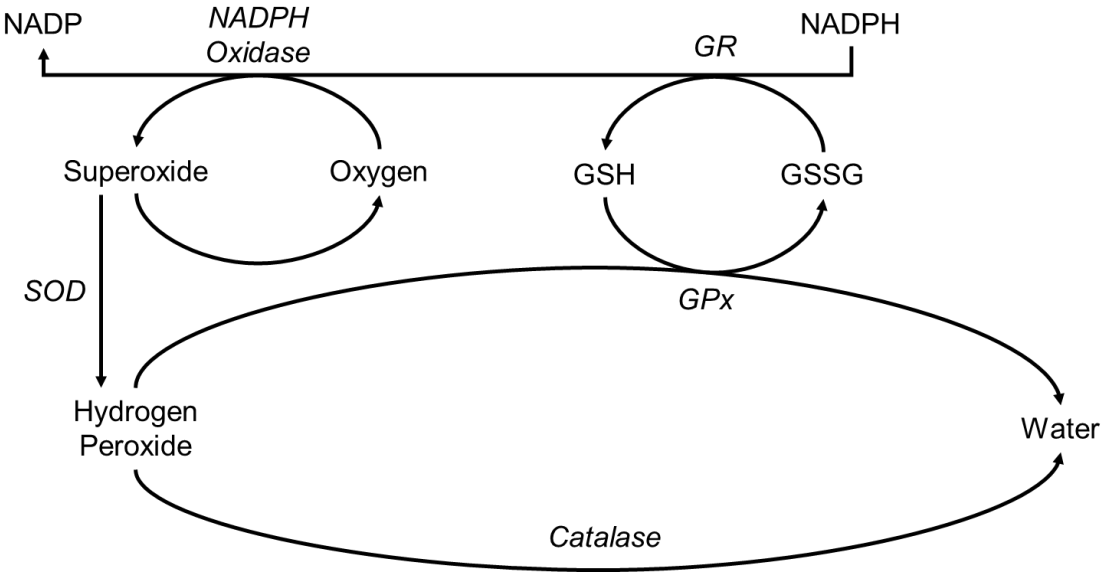
2.4.1.3 Glutathione

Glutathione (γ-glutamyl-cysteinyl-glycine; GSH) is a low-molecular-weight thiol (0.5-10mmol/L) and encompasses the primary redox couple in human cells; glutathione (GSH): glutathione disulfide (GSSG). Glutathione is most abundant within the cytosol of human cells (1-5mM; Newsholme *et al.*, 2012), however, it does exist within subcellular compartments such as the mitochondria and peroxisomes (Lu, 2000); this compartmentalisation determines the functionality of glutathione. Mitochondrial glutathione's primary function is to modulate apoptosis/necrosis (Yuan & Kaplowitz, 2008), whereas nuclear glutathione has a cardinal role within the cell division process (Pallardo *et al.*, 2008). With that being said, glutathione's functionality relies on its ability to scavenge hydrogen peroxide, and organic hydroperoxides, through the 5-

member glutathione peroxidase (GPx1-GPx5) family of tetrameric enzymes; except for the monomeric structure of GPx4 (Moran & Cortazar, 2012)

In tandem, the reducible two FAD molecules with glutathione reductase (GR), synthesises glutathione disulfide back to the sulfhydryl form, with the reducing power coming from NADPH ($GSSG + NADPH + H^+ \leftrightarrow 2GSH + NADP^+$) (Kabel, 2014). An overview of this pathway is depicted in Figure 2.12. Interestingly within mitochondria, GSH cannot be synthesised, and therefore, relies on electroneutral exchange from the cytosol via oxoglutarate, and dicarboxylate mitochondrial carriers for cell survival (Griffith & Meister, 1985).

Figure 2.12. A simplified overview of the role of GSH:GSSG in the scavenging of hydrogen peroxide. GSH undergoes a redox reaction using glutathione peroxidase GPx to detoxify ROS like hydrogen peroxide (H2O2). The main source of H2O2 is from the conversion of superoxide anion (O2-·) by the enzymatic action of superoxide dismutase (SOD). GSH is converted to an oxidized form (GSSG) and is recycled back to GSH by the enzymatic reaction of glutathione reductase (GR) which requires the cofactor nicotinamide adenine dinucleotide phosphate (NADPH) to form a redox cycle.



With regards to the glutathione mechanism of action, the enzymatic active site contains a selenoprotein and selenocysteine residue, which undergo a redox cycle using selenol (E-SeH) to reduce hydrogen peroxide (10^7 $M^{-1}s^{-}1$) and organic peroxides (Winterbourn, 2013); in turn, producing seleneyl derivatives. Once the hydrogen peroxide has become reduced, the seleneyl sulfide adduct resynthesises the active form of the enzyme with water being produced in the process (Patlevic *et al.*, 2016).

Glutathione reductase is equally as important as its peroxidase counterpart, as it regulates the ratio of GSH:GSSG, in favour of the former, which is critical to cell survival in normal healthy individuals (Jozefczak *et al.*, 2012). This is evident in the upregulation of signalling pathways associated with a reduction in cell proliferation and apoptosis (Flohe, 2013). Additionally, disruptions to the GSH:GSSG redox state have been implicated in the etiology and pathophysiology of a number of diseases (Reynolds *et al.*, 2007; Valko *et al.*, 2007).

Some evidence would also point to glutathione's ability to act as an electron sink (Winterbourn, 1993). To clarify, in the presence of free radicals, GSH can act as a substrate for hydroperoxide reduction; in turn generating a sulfur-centered radical which is subsequently detoxified through SOD as outlined below;

$$GS^{\bullet} + GSH \rightarrow GSSG^{\bullet-} \quad [8 \times 10^{8}\ M^{-1}s^{-1}]$$

$$GSSG^{\bullet-} + O_2 \rightarrow GSSG + O_2^{\bullet-} \quad [1.6 \times 10^{8}\ M^{-1}s^{-1}]$$

Although both kinetically, and thermodynamically sound, the role of GSH (and subsequent enzymatic dismutation of SOD), to act as an electron sink is controversial and the evidence is scarce (Forman *et al.*, 2013).

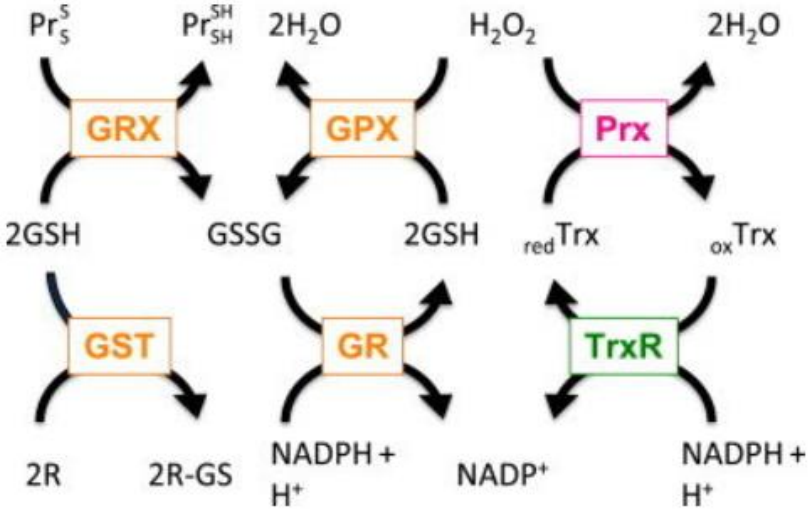
2.4.1.4 Thioredoxin

Thioredoxin is a ubiquitous disulfide reductase system comprising of the enzymes thioredoxin and thioredoxin reductase, which serve to maintain a pool for electrons to be transferred during DNA synthesis and protection against oxidative stress (Nordberg & Arner, 2001). The thioredoxin system contains two major pathways; cytosolic Trx1 plays an essential role in redox regulation and NO· signalling (Holmgren, 2000), while the mitochondrial-bound Trx2 is needed for mitochondria-dependent apoptosis and cell growth (Tanaka *et al.*, 2002).

The antioxidant capacity of thioredoxin comes from its ability to transfer electrons to peroxiredoxins and methionine sulfoxide reductases. Thioredoxins, in synergy with the glutathione system, aims at removing RONS; thus, exists a compensatory cross-talk between the two antioxidant systems (Lu & Holmgren, 2014). A schematic representation can be observed in Figure 2.13. Although thioredoxin is present in the plasma and can exert antioxidant properties through the removal of hydrogen peroxide, it is thought that it serves a back up to glutathione by providing

additional thiol buffering capacity; this has been suggested due to the relatively low concentration of thioredoxin (6 nM) compared to glutathione (1 µM) present in the plasma (Nakamura *et al.*, 1997; Berntsen *et al.*, 1998). It is clear the thioredoxin system has a key responsibility in cellular antioxidant capacity, and this is only reiterated in the high concentrations observed in pathophysiology such as malignant disease, HIV, and cardiac conditions (Burke-Gaffney *et al.*, 2005).

Figure 2.13. The mutual interplay between the thioredoxin, peroxiredoxin, and glutathione antioxidant systems. Hydrogen peroxide (H2O2) can be reduced by peroxiredoxins (Prx) or glutathione peroxidases (GPX), which couple reduction of H2O2 with oxidation of glutathione (GSH). Oxidized Prx can be reduced by thioredoxins (Trx). Subsequently, oxidized Trx become reduced by thioredoxin reductase (TrxR) in a NADPH-dependent manner. Oxidized glutathione disulfide (GSSG) is reduced by glutathione reductase (GR) in the presence of NADPH. Further, glutaredoxins (Grx) can reduce disulfide (S-S) bonds in proteins (Pr), and glutathione S transferase (GST) is using GSH to conjugate and thus to detoxify reactive electrophilic compounds (R).



From Espinosa-Diaz et al. (2015).

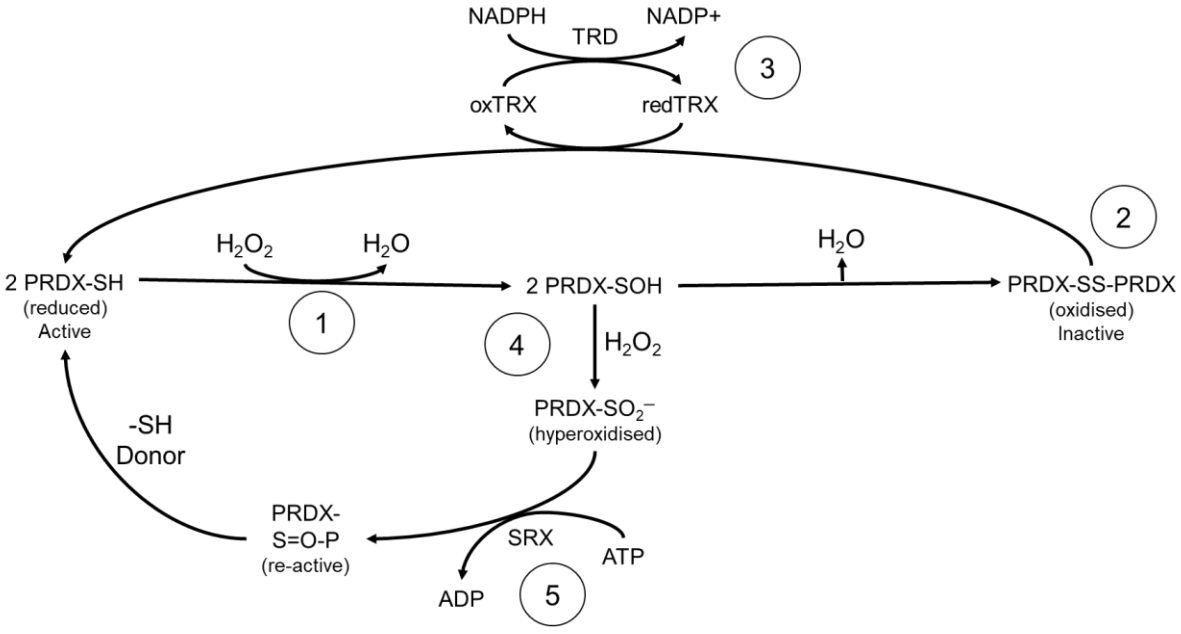
2.4.1.5 Peroxiredoxins

Peroxiredoxins are a large family of ubiquitous peroxidases, capable of sequestering hydrogen peroxide, alkyl hydroperoxides, and peroxynitrite; however, unlike glutathione and thioredoxin, peroxiredoxin requires no co-factors for their reducing power (Perkins *et al.*, 2015). Together, peroxiredoxins, thioredoxins and glutathione work synergistically to act as a guardian against oxidative stress, and regulatory processes as depicted in Figure 2.14.

Although peroxiredoxins have evidence for their ability to protect against oxidative damage (Fisher, 2011), it would seem excessive for cells/organisms to have multiple enzymes to remove peroxides (Rhee, 2016); namely, catalase, glutathione peroxidase, ascorbate peroxidase, and indeed peroxiredoxins. To elaborate, not only are peroxiredoxins recognised for their antioxidant activity, but much of the research has focused on peroxiredoxin as a modulator of peroxide concentration to stress and non-stress processes; specifically, signalling processes linked to growth factors, angiogenesis, and cytokine signalling (Oakley *et al.*, 2009; Finkel, 2011).

Located throughout cellular compartments (nuclei, mitochondria, cytosol, endoplasmic reticulum; Rhee *et al.*, 2005), peroxiredoxin functions to excite several divergent physiological functions, including apoptosis, lipid metabolism, protection of genomic instability, and cellular homeostasis (Fujii & Ikeda, 2002). Uniquely, depending on the context, peroxiredoxins may also inhibit, or promote the development of cancer tumour growth (Park *et al.*, 2016).

Figure 2.14. The primary antioxidant pathway of the peroxiredoxin system.



Peroxiredoxins scavenge hydrogen peroxide (1), which is subsequently reduced by the thioredoxin system (2). Peroxiredoins also become hyperoxidised to prevent toxic effects (4,5). SH group donors such as GSH and/or TRX reduce the hyperoxidised peroxiredoxin back to its active form. Adapted from O'Flaherty (2014).

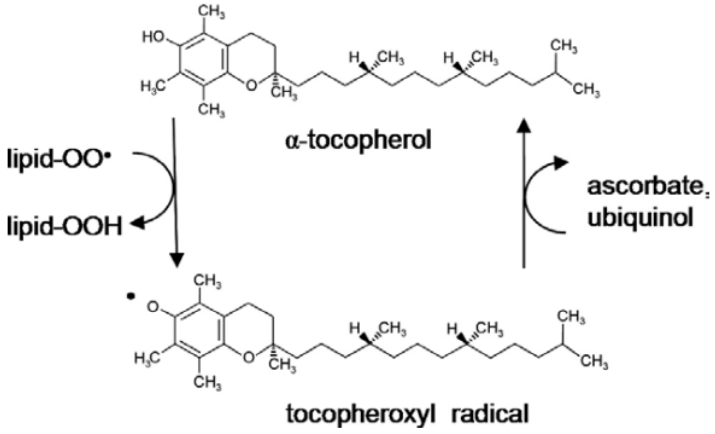
2.4.2 Non-Enzymatic Antioxidants

2.4.2.1 Vitamin E

Vitamin E is the name given to the lipid-soluble, chain-breaking micronutrients comprised of two subfamilies; the α-, β-, γ-, δ-tocopherols which contain a phytyl tail and three chiral centres, and the α-, β-, γ-, δ-tocotrienols, each with relatively similar antioxidant potencies (Zingg, 2007). Although the free radical scavenging activity are similar, there are clear molecular differences with regards to reactivity within the tocopherols; α > γ > β > δ (Azzi & Stocker, 2000). Alpha-tocopherol has the highest rate constant (k_1 = 2.35 x 10^6 M^{-1} s^{-1} at 30°C) of all the tocopherols for hydrogen abstraction (Burton & Ingold, 1983), and has the highest bioavailability. Thus, for the purpose of this thesis, research presented will focus on the α-tocopherol moiety unless stated otherwise.

Alpha-tocopherols antioxidant function lies in its ability to inhibit lipid peroxidation by sacrificing an electron; in turn, generating the α-tocoheroxyl free radical (Leonard *et al.*, 2004; Richelle *et al.*, 2004). This reaction occurs at a rate several orders of magnitude than that of the lipid peroxyl radical during the propagation phase of lipid peroxidation (Dutta & Dutta, 2003). The regeneration of α-tocopherol by ascorbate and ubiquinol-10 (Schneider, 2005), has led to a plethora of investigations using vitamin C and vitamin E tandem interventions (Tucker & Townsend, 2005). The ability of ascorbate to regenerate tocopherol is based on the premise of ascorbates redox potential (280 mV) in contrast to that of tocopherol (500 mV) which can be summarised in Figure 2.15.

Figure 2.15. Schematic overview of the resynthesis of α-tocopherol with ascorbate acting as the reductant [2 × 10^5 $M^{-1}s^{-1}$].



Taken from Tucker & Townsend (2005).

Alpha-tocopherol is abundantly located within the membranes of cells and subcellular organelles where it can exert its antioxidant effect by quenching lipid-targeted free radical damage (Liu *et al.*, 2002). Upon interaction with a lipid peroxidative free radical, the newly formed tocoperoxyl radical can undergo several further reactions; (i) propagating further lipid peroxidation, (ii) the downstream production of tocopheryl quinones, (iii) the generation of unreactive tocopherol dimers, or (iv) the resynthesis of tocopherol. These reactions can be summarised below (Rathore *et al.*, 2011):

1. $ROO\cdot + \alpha\text{-}TOH \rightarrow ROOH + \alpha\text{-}TOH\cdot$ [10^5 - 10^6 $M^{-1}s^{-1}$]
2. $\alpha\text{-}TOH\cdot + CoQ_{10}H_2 \rightarrow \alpha\text{-}TOH + CoQH\cdot$

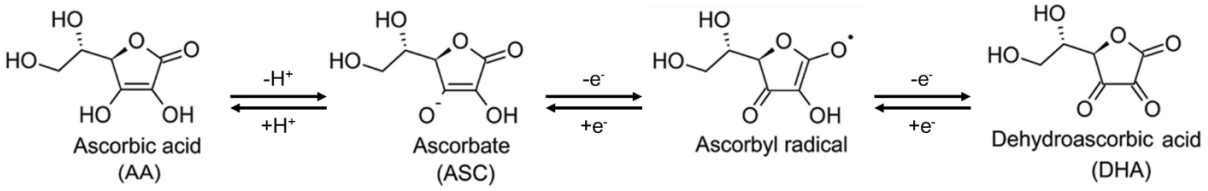
   $CoQH\cdot + e^- \rightarrow CoQ$
3. $ROO\cdot + \alpha\text{-}TOH\cdot \rightarrow$ Non-Radical Products
4. $\alpha\text{-}TOH\cdot + RH$ (PUFA) $\rightarrow \alpha\text{-}TOH + R\cdot$ (Alkyl Radical) [1×10^{-1} $M^{-1}s^{-1}$]

   $R\cdot + O_2 \rightarrow ROO\cdot$ (PUFA-Dervied Peroxyl Radical)

2.4.2.2 Vitamin C

Vitamin C (ascorbic acid, ascorbate, and dehydroascorbic acid) is a cytoplasmic, water soluble antioxidant with the ability to sequester superoxide and hydroxyl radicals (Li *et al.*, 2010). Due to its water-soluble properties, the biological cells lose approximately 3% of its ascorbic acid content per day (Delanghe *et al.*, 2007). Moreover, ascorbic acid recycles α-tocopherol. Once it has been involved in oxidation, other reducing agents such as, glutathione, alpha-lipoic acid, nicotinamide adenine dinucleotide (NADH), and NADPH, can resynthesise it back to ascorbic acid (Tripathi *et al.*, 2009).

Figure 2.16. Schematic overview of the reactions associated with ascorbate metabolism.



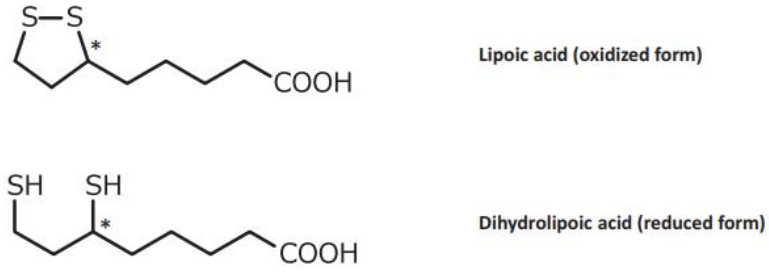
Ascorbic acid can exhibit a pro-oxidant effect under certain circumstances. To clarify, oxidation of ascorbate generates a relatively unreactive ascorbyl free radical (Traber & Stevens, 2011). However, the ascorbyl free radical has poor reactivity partly

due to the localisation of the unpaired electron in the molecular orbital (Halliwell, 2001). Additionally, evidence from *in vivo* models suggest that ascorbate has the pro-oxidant potential to reduce Fe^{3+} to Fe^{2+} (Powers *et al.*, 2004); subsequently increasing the likelihood of downstream superoxide production (Bowry *et al.*, 1995). Ascorbate can also produce hydroxyl radical in the presence of transition metals and hydrogen peroxide by Fenton chemistry (Pompella *et al.*, 2003).

2.4.2.3 Alpha-Lipoic Acid

Alpha-lipoic acid (ALA; $C_8H_{14}O_2S_2$) is an organosulfur that forms an intramolecular disulfide bond in its oxidised form, which also exists as dihydrolipoic acid in its reduced form (DHLA) as illustrated in Figure 2.17.

*Figure 2.17. Structure of ALA (A), and its reduced form, dihydrolipoic acid (B); * the structure contains a chiral centre.*



ALA and DHLA have been shown to act as biological antioxidants and modulate the intracellular thiol redox status (Rochette *et al.*, 2015). The ratio of ALA:DHLA is often portrayed as the 'universal antioxidant' due to its potent redox potential; -320 mV. When contrasted to the GSH:GSSG redox potential of approximately -240 mV, it would suggest that DHLA offers superior protection from RONS-mediated oxidative damage. Additionally, this ALA:DHLA couple can also resynthesis other cellular antioxidants such as GSH, ascorbic acid, and vitamin E (Marangon *et al.*, 1999); however, unlike ascorbate, DHLA possesses the remarkable ability to resist transforming into a free radical itself upon sequestration of RONS (Petersen Shay *et al.*, 2008).

Early research by Packer and colleagues (1995) demonstrated the ability of ALA:DHLA to quench the hydroxyl radical, singlet oxygen and hypochlorous acid (Kaiser *et al.*, 1989; Scott *et al.*, 1994; Vriesman *et al.*, 1997; Trujillo and Radi, 2002). Additionally, others have observed a reduction in $NO\cdot/O_2\cdot^-$-driven production of

peroxynitrite, despite negligible outcomes associated with *in vivo* models (Trujillo and Radi, 2002).

On an experimental level, supplementation of ALA has been used in several murine and human studies to examine the effect on oxidative stress. For example, supplementation of 600mg/day for a period of 8 weeks, decreased biomarkers associated with lipid peroxidation in humans (Marangon *et al.*, 1999). Similarly, in the murine model, 150 mg/kg body wt/day for a total of 8 weeks resulted in an attenuation of exercise-induced lipid peroxidation across a variety of tissue types (Khanna *et al.*, 1999). It should be emphasised, there is a plethora of evidence showing similar conclusions across a multitude of tissue types, and (patho)physiological outcome measures associated with oxidative stress (Hagen *et al.*, 2000; Arivazhagan & Panneerselvam, 2000; Arivazhagan *et al.*, 2001; Packer *et al.*, 2001; Fogarty *et al.*, 2013). Paradoxically, several reports have demonstrated pro-oxidant mechanisms associated oxidative tissue damage, transition metal-driven hydroxyl generation and lipid peroxidation following ALA and/or DHLA administration (Romero *et al.*, 1992; Slepneva *et al.*, 1995; Morkunaite-Haimi *et al.*, 2000; Lyublinskaya *et al.*, 2014).

2.4.2.4 Carotenoids

The term ‘carotenoids’, characterises the ubiquitous fat-soluble disposition of over 600 pigments synthesised by plants, fungi, and algae micro-organisms (Stahl & Sies, 2003). Evidence would suggest carotenoids play accessory roles within immune system function, signalling pathways (Bertram, 1993), apoptosis and cell differentiation (Krinsky, 1993), along with other significant physiological processes including antioxidant defence (Palozza *et al.*, 2009; Young & Lowe, 2018).

Although it has been suggested over 600 carotenoids exist, it is thought that the human diet consists of approximately 10% of these with only 20 being present within blood and tissue (Khachik, 2006; Parker, 1989). The most prominent carotenoids within human plasma include α-, β-, γ-, and ζ-carotene, lycopene, lutein, neurisporene and zeaxanthin (Breecher & Khachik, 1992); often residing within the lipid bilayer or perpendicular to the membrane (Wisniewska & Subczynski, 1998; Wisniewska & Subczynski, 2006).

The antioxidant mechanisms associated with carotenoids can be characterised as 4 chemical reactions;

(i) Oxidation $Car + R^{\cdot +} \rightarrow Car^{\cdot +} + R$

(ii) Reduction $Car + e^{-} \rightarrow Car^{\cdot +}$

(iii) Hydrogen Abstraction $Car[H] + R^{\cdot} \rightarrow Car^{\cdot} + RH$

(iv) Hydrogen Addition $Car + ROO^{\cdot} \rightarrow [Car\text{-}ROO]^{\cdot}$

$[Car\text{-}ROO]^{\cdot} + ROO^{\cdot} \rightarrow ROO\text{-}Car\text{-}ROO$

Due to the multifactorial nature of the endogenous antioxidant system, some of the research surrounding carotenoids has focused on their ability to network with other antioxidant compounds. To elaborate, the redox potentials of carotenoids can range from 980-1060 mV and thus are able to abstract a hydrogen/electron from ascorbic acid or α-tocopherol. This premise has mixed support as some would state carotenoids protect α-tocopherol (Bohm *et al.*, 1997), whereas others would conclude carotenoids use α-tocopherol and ascorbic acid to resynthesis (Mortensen & Skibsted, 1997; Valgimigli *et al.*, 1997).

2.4.2.5 Polyphenols

Polyphenols characterises the family of over 8000 ubiquitous antioxidants which are abundantly found within plant food products (Pandey & Pizvi, 2009); their commonly shared phenolic hydroxyl group(s) provide the premise for their antioxidant properties (Frankel *et al.*, 1993; Rice-Evans *et al.*, 1996; Rice-Evans *et al.*, 1997). Due to their profusion of health benefits, there is a plethora of evidence examining the effects of polyphenols on cardiovascular disease (Surh, 2003; Scalbert *et al.*, 2005; Kuriyama *et al.*, 2006; Schewe *et al.*, 2008).

Polyphenolic compounds can exhibit both direct and indirect antioxidant effects; the former relating to direct scavenging and subsequent consumption of the antioxidant, in contrast to the influencing/activating of redox pathways associated with indirect antioxidant activity (Dinkova-Kostova & Talalay, 2008). The outcomes associated with polyphenolic-mediated antioxidant activities are contentious; this is primarily due to the large variations of polyphenol concentrations used between research models (Scalbert & Williamson, 2000; Halliwell *et al.*, 2005; Vauzour *et al.*, 2010). Secondly, many direct antioxidants operate on a competitive nexus (Hu, 2011); and as such are likely to outmatch polyphenols on a competitive-concentration basis (Dieber-Rotheneder *et al.*, 1991; Wen *et al.*, 1996; Lee *et al.*, 1997; Ness *et al.*, 1999). The more plausible explanation of polyphenolic antioxidant properties relates to how

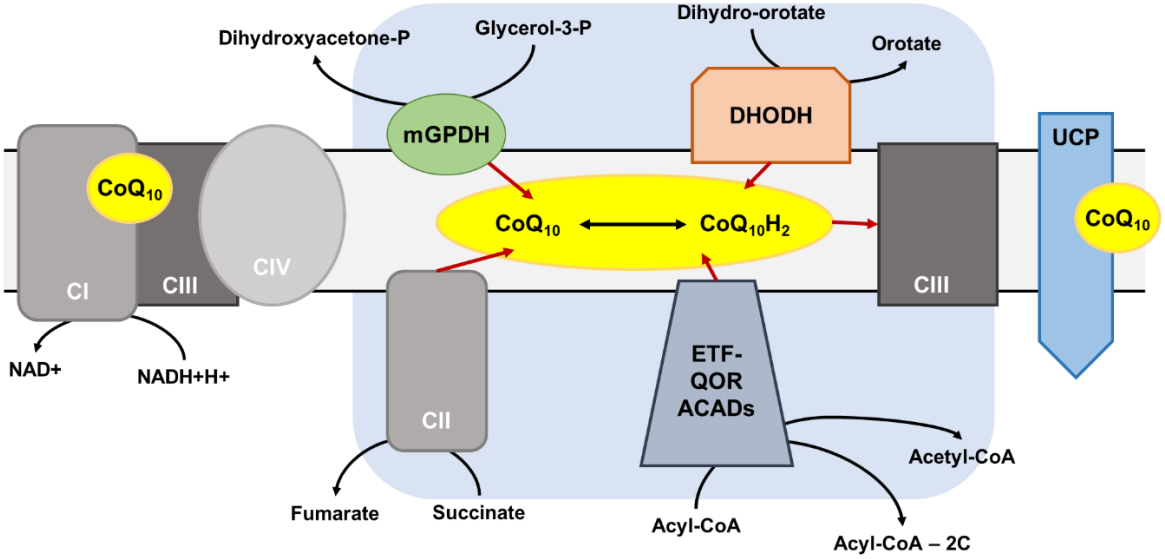
they interact with enzymatic antioxidant expression and cytoprotective proteins; for example, SOD, CAT, thioredoxin, GPx, hemeoxygenase-1 and NADPH:quinone oxidoreductase-1 (Motohashi & Yamamoto, 2004; Chen & Kong, 2004; Pan, 2008; Halliwell, 2008). With specific interest to polyphenol supplementation and beneficial effects associated with exercise performance or oxidative stress, the literature remains equivocal (Myburgh, 2014). It appears the evidence is insufficient to make recommendations for endurance performance; similarly, there is insufficient evidence to suggest polyphenolic compounds, either supplementation or food produce, should be avoided (MaCrae & Mefferd, 2006; Cureton *et al.*, 2009; Davis *et al.*, 2010; Nieman *et al.*, 2010; Kressler *et al.*, 2011). It is thought this could be associated with exercise-induced excess radical generation, neutrophil oxidative burst, breakdown of myoglobin, and oxidative damage to lipids and proteins (Goldfarb *et al.*, 2011; Myburgh, 2014).

2.4.2.6 Coenzyme Q10

Coenzyme Q10 (ubiquinone) is a benzoquine (2,3-dimethoxy-5 methyl-6-decaprenyl-benzoquinone) found within almost every cell of the human body (Turunen *et al.*, 2004), and is synthesised endogenously from the acetyl-CoA-mediated mevalonate cycle (Bentinger *et al.*, 2010).

The primary physiological function of Coenzyme Q10 is to act as an electron transporter within the mitochondrial transport chain specifically, between the complexes NADH dehydrogenase to succinate dehydrogenase, and from succinate dehydrogenase to the cytochrome bc complex (Bentinger *et al.*, 2010). It achieves this due to its oxidoreductase properties whereby upon accepting electrons it exists as ubiquinol in contrast to when it transfers electrons to the target downstream complexes where it returns to its oxidised form of ubiquinone (Molyneux *et al.*, 2008). It has been suggested the efficiency and stability of these electron transport chain supercomplexes prevent this electron leakage and subsequent production of ROS (Genova & Lenaz, 2014; Milenkovic *et al.*, 2017; Hernandez-Camacho *et al.*, 2018). An overview of the primary functions of coenzyme Q10 associated with the mitochondria are illustrated in Figure 2.18.

Figure 2.18. An overview of the mitochondrial functions of CoQ10. The main function of CoQ_{10} in mitochondria is to transfer electrons to CIII. By transferring two electrons to CIII, the reduced form of CoQ_{10} is oxidized to ubiquinone. The pool of ubiquinol can be restored by accepting electrons either from members of the electron transport chain (CI and CII), GPDH and DHODH that use cytosolic electron donors, or from ACADs. CoQ_{10} is also a structural component of both CI and CIII and is associated with respiratory supercomplexes, especially the depicted supercomplex I+III+IV. CoQ_{10} is an obligatory factor in proton transport by UCPs with concomitant regulation of mitochondrial activity.



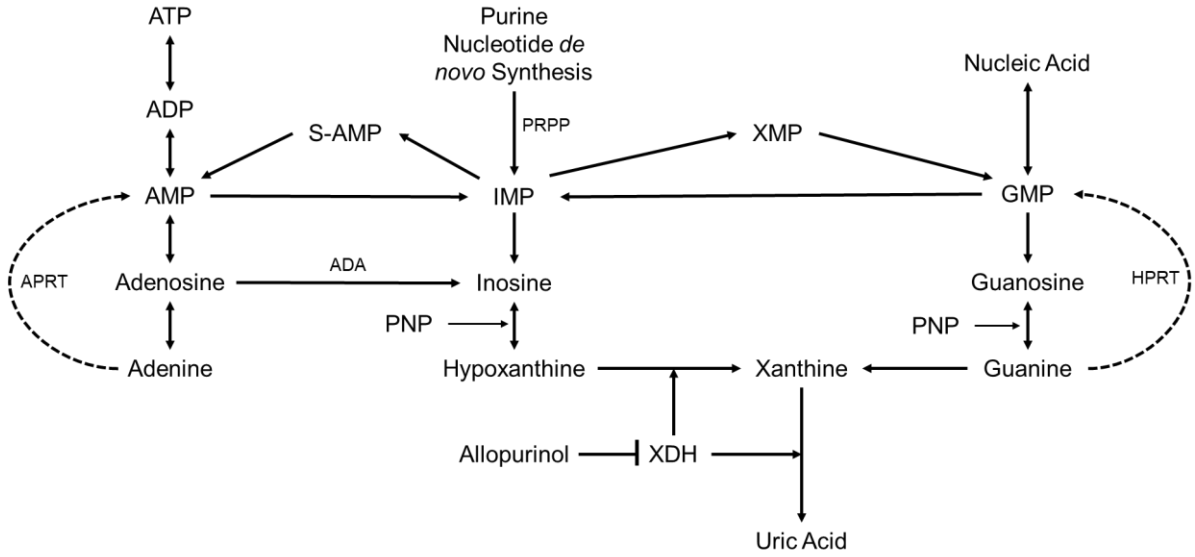
Abbreviations – C, complex; GPDH, glycerol-3-phosphate dehydrogenase; DHODH, dihydroorotate dehydrogenase; ACADs, acyl-CoA dehydrogenases; UCP, uncoupling proteins; ETF-QOR, electron transferring flavoprotein:ubiquinone oxidoreductase. Adapted from Hernandez-Camacho et al., (2018).

Coenzyme Q10 can exert a number of direct and indirect antioxidant properties within the cell. Directly, it can participate in the attenuation in RONS-induced damage to DNA and lipid peroxidation by scavenging free radicals (Sugiyama *et al.*, 1980; Yasukazu *et al.*, 2006). Additionally, it can also contribute indirectly to redox balance by preventing excess calcium accumulation (Casagrande *et al.*, 2018) and regenerating tocopherol (Martin *et al.*, 2007). Coenzyme Q10 ingestion is beneficial for cardiovascular disease (Flowers *et al.*, 2014), neurodegenerative diseases (Asencio *et al.*, 2016), Parkinson's (Liu *et al.*, 2011), diabetes (Fan *et al.*, 2017), and in chronic fatigue syndrome (Campagnolo *et al.*, 2017).

2.4.2.7 Uric Acid

Uric acid ($C_5H_4N_4O_3$; 7,9-dihydro-1H-purine-2,6,8(3H)-trione) is the final metabolic product associated with purine degradation, catalysed by xanthine oxidoreductase (Harrison, 2002); the overarching metabolic processes can be observed in Figure 2.19.

Figure 2.19. Purine metabolism in humans. Pertinent pathways: formation of the purine nucleotide inosine monophosphate (IMP) from non-purine precursors (synthesis de novo), or purine bases (salvage reactions); purine nucleotide interconversion reactions; degradation to the end product uric acid (catabolic reactions).



Abbreviations: ADA, adenosine deaminase; APRT, adenosine phosphoribosyl transferase; HPRT, hypoxanthine posphoribosyl transferase; PNP, purine nucleoside phosphorylase; PRPP, phosphoribosyl pyrophosphate synthase; XDH, xanthine dehydrogenase. Adapted from Simoni et al., (2007).

Uric acid has the ability to exhibit antioxidant properties associated with oxidative attack to erythrocyte lipid membranes (Kellogg & Fridovich, 1977; Ames *et al.*, 1981). With that being said, a large majority of research focuses on the ability of uric acid to provide protection to the central nervous system (Hooper *et al.*, 1998; Duan *et al.*, 2002; Spitsin *et al.*, 2002; Amaro *et al.*, 2007). It has been estimated that uric acid accounts for approximately 60% (200 - 400 µmol/l; 3.4 - 6.8 mg/dl) of human plasma antioxidant capacity (Maiuolo *et al.*, 2016). It has also been postulated that uric acid may chelate transition metals within serum, thus stabilising ascorbic acid (Davies *et al.*, 1986; Einsele *et al.*, 1987; Miura *et al.*, 1993). Whiteman and colleagues suggest uric acid may attenuate the decomposition of peroxynitrite (Whiteman *et al.*, 2002),

however this theory has faced controversy due to the higher rate constant of carbon dioxide reacting with peroxynitrite (Squadrito *et al.*, 2000).

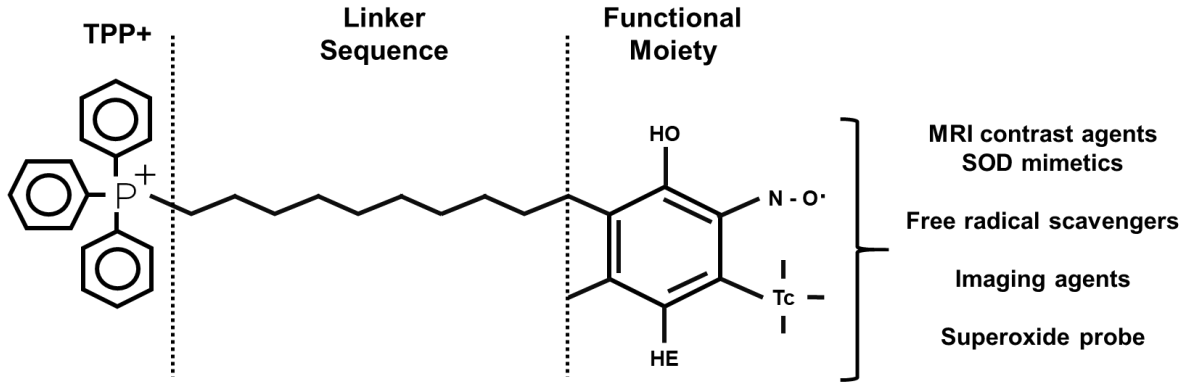
The current body of literature would also propose a pro-oxidant action of uric acid due to the generation of the urate radical upon reaction with oxidising agents; however, the physiological significance of this within *in vivo* models is questionable as the unpaired electron is delocalised to the purine ring (Shamsi & Hadi, 1995). Finally, it has been suggested uric acid-derived radicals, or indeed uric acid itself, increases the generation of NADPH oxidase-dependent free radicals (Sautin & Johnson, 2010). Thus, the antioxidant-oxidant paradox associated with uric acid still hasn't been fully elucidated.

2.4.3 Mitochondrial-Targeted Antioxidants

As indicated, mitochondria are vulnerable to oxidative damage as they are a primary source of RONS (Balaban *et al.*, 2005). Consequently, the excessive accumulation of oxidative damage can cause mitochondrial dysfunction (Smith & Murphy, 2010), and disease, including Alzheimer's (Swerdlow, 2011), Parkinson's, Friedreich's ataxia (Reddy, 2008), atherosclerosis (Victor *et al.*, 2009), and type I diabetes (Maiese *et al.*, 2007). It has been proposed that this mitochondrial damage can propagate reactions which initiate cell apoptosis; resulting in organ failure and disease (Sheu *et al.*, 2005). Additionally, Brookes *et al.* (2004), postulates that changes in mitochondrial calcium, ATP, and RONS metabolism, are common hallmarks of diseases associated with mitochondrial dysfunction. To expound, in chemotherapy-induced apoptosis, it is known that mitochondria produce excessive amounts of superoxide resulting in the oxidation of the inner membrane lipid cardiolipin (Decaudin *et al.*, 1998). Consequently, this causes a disturbance to the mitochondrial transmembrane potential, resulting in a decreased function of the inner and outer mitochondrial membranes. In myocardial ischemia-reperfusion injury, an excessive calcium overload can produce RONS, causing a disturbance in the mitochondrial permeability-transmembrane pore (Sheu *et al.*, 2005). As a result, it has been hypothesised if a compound can attenuate excessive mitochondrial calcium accumulation, RONS generation, and improve mitochondrial energy production, then the potential for treating the aforementioned diseases (or more probable, the symptoms) is highly desirable.

In mitochondria, the outer membrane is relatively permeable, however, the negative potential of the proton gradient (150-180 mV) of the inner mitochondrial membrane makes it difficult to deliver compounds to the mitochondria. As a result, lipophilic cations have been developed to work in conjunction with this gradient; this was first pioneered by Robin Smith and Michael Murphy (Murphy, 1997; Murphy & Smith; 2000; Smith *et al.*, 2003). By using lipophilic cations, Sheu *et al.* (2005) estimated that accumulation of specific compounds can increase 100- to 1000-fold within the matrix of the mitochondria. This is largely due to the 61.5-mV hyperpolarisation in accordance with the Nernst equation, allowing for the diffusion and accumulation of lipophilic monovalent cations (Ross *et al.*, 2005). As such, cationic compounds were developed to exploit this mitochondrial quality, namely rhodamine-123 and triphenylphosphonium (TPP+) (Smith *et al.*, 2012). TTP+ is one of the most commonly used cations as it is driven by a large mitochondrial membrane potential, allowing for a rapid accumulation of compound within the mitochondrial matrix (Finichiu *et al.*, 2015). The application of TPP+-conjugated bioactive molecules have been developed to deliver antioxidants, probes, and other pharmacological agents to the mitochondria; these include coenzyme Q10, metformin, and vitamin E (Cheng *et al.*, 2016; Asin-Cayuela *et al*., 2004; Skulachev *et al*., 2010). The chemical modification of TPP+-conjugated molecules is outlined in Figure 2.20.

Figure 2.20. The chemical modifications and applications associated with TPP+ bioactive molecules.



Adapted from Zielonka et al., (2017).

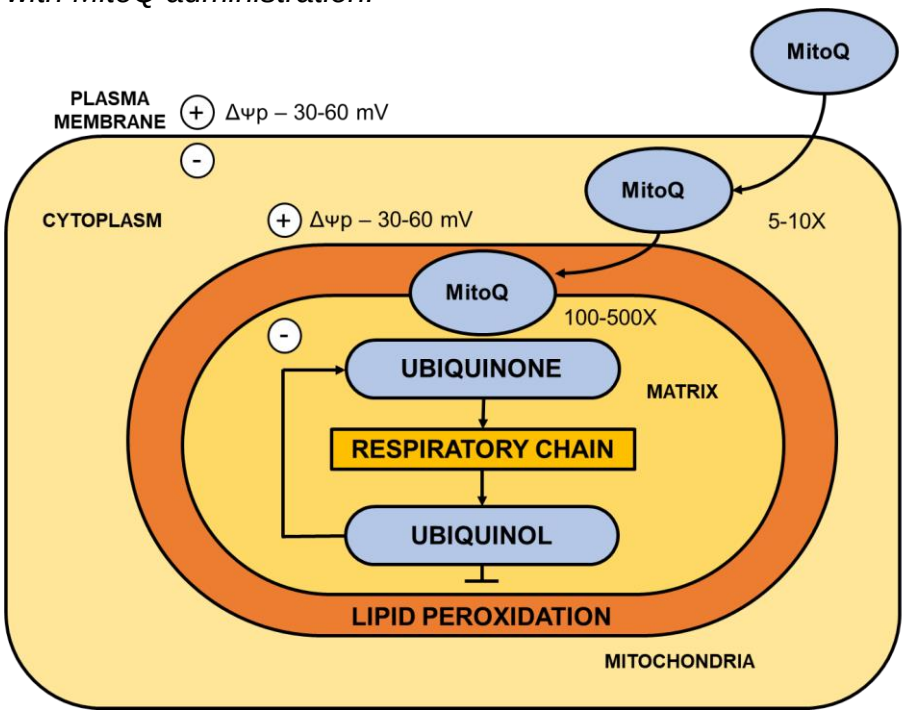
In relation to the efficacy of mitochondrial-targeted antioxidants, there is a greater protective effect as these targeted antioxidants accumulate in mitochondria, compared to general cytosolic based antioxidants (i.e. ascorbate). In support Oyewole

et al. (2014), demonstrated greater protection of mitochondrial DNA with the mitochondrial-targeted antioxidant MitoQ in human skin fibroblasts when exposed to hydrogen peroxide and UV-radiation, in comparison to *N*-acetyl-cysteine, resveratrol and curcumin. Due to the exponential use and novel development of mitochondrial-targeted compounds, a comprehensive review of each compound is beyond the scope of this thesis. As such only the main compound of interest to this thesis is outlined below.

2.4.3.1 MitoQ

MitoQ is a mitochondrially targeted derivative of ubiquinone; linked to a TPP+ cation with a 10-carbon alkyl chain (Ostojic, 2016). It is rapidly taken up by mitochondria, and distributed to the matrix face of the inner mitochondria membrane. Once mitochondrial saturation is achieved, MitoQ is continually recycled to the active quinol antioxidant by succinate dehydrogenase (Smith & Murphy, 2010). Ubiquinone has a plethora of physiological properties including maintenance of the mitochondrial electron transport chain, and sequestering of lipid peroxyl radicals and peroxynitrite (Murphy, 2008). An overview of the transport and mechanisms of action of MitoQ shown below (Figure 2.21).

Figure 2.21. Schematic overview of the absorption and primary mechanisms of action associated with MitoQ administration.



Adapted from Broome et al. (2018).

To date, much of the research involving MitoQ has been conducted within animal models, while clinical research remains limited (see Table 2.5).

Table 2.5. Overview of studies associated with MitoQ and outcome parameters associated with muscle function.

Parameter	Model	Intervention	Supplement	Outcome
Mitochondrial biogenesis and function	Humans	Exercise	10mg/day	No effect*
	Rats	High-fat diet	375 µmol/kg for 8 weeks	Increased mitochondrial function┼
Insulin sensitivity	Rats	High-fat diet	375 µmol/kg for 8 weeks	Increased glucose tolerance┼#
Vascular function	Humans	Aged population	20mg/day for 6 weeks	Improved aged-related vascular function¥
Ageing	Mice	Aged population	100 µmol for 15 weeks	No effectΔ

** Shill et al., 2016; ┼ Coudray et al., 2016; # Feillet-Coudray et al., 2014; ¥ Rossan et al., 2018; Δ Vays et al., 2014*

In addition, Snow and colleagues (2010), investigated MitoQ to slow the progression of Parkinson's disease over a 12-month period. Following the experimental phase (10mg/day for 12-months), authors concluded that MitoQ failed to attenuate Parkinson's disease progression; this may be due to the fact that by the time Parkinson's is clinically diagnosed it has been estimated 50% of the neurons are lost and the remaining, viable neurons are beyond the stage which cell death can be prevented (Smith & Murphy, 2010). Secondly, evidence surrounding MitoQ and neuro-penetration, specifically to the brain, is scarce in the humans (Smith, 2003; Rodriguez-Cuenca *et al.*, 2010). MitoQ has also been examined in type 2 diabetes. Escribano-Lopez and colleagues (2016) reported an increase in GPx1 concentration with TNF-α and a reduction in NF-κβ following MitoQ administration; concurrently, generation of mitochondrial ROS was reduced in the MitoQ group. Finally, Rossman *et al.*, (2018) demonstrated an improvement in endothelial function and reduced aortic stiffness in healthy older adults following 6 weeks of MitoQ supplementation at 20 mg/day.

To date, only one study has been published examining exercise and MitoQ supplementation. Shill and colleagues (2016) showed that exercise increased circulating angiogenic cells (CACs); resulting in the maintenance of vascular integrity and endothelial repair. However, MitoQ had no effect on CAC-related outcomes, lipid peroxidation, muscle oxidative capacity, and $\dot{V}O_{2max}$. A limitation of this study is aligned to the dosing protocol. Although the manufacturers recommend 10mg/day, research suggests that a minimum of 20mg/day is required (Smith & Murphy; 2010).

2.4.4 Dietary Food Consumption and Oxidative Stress

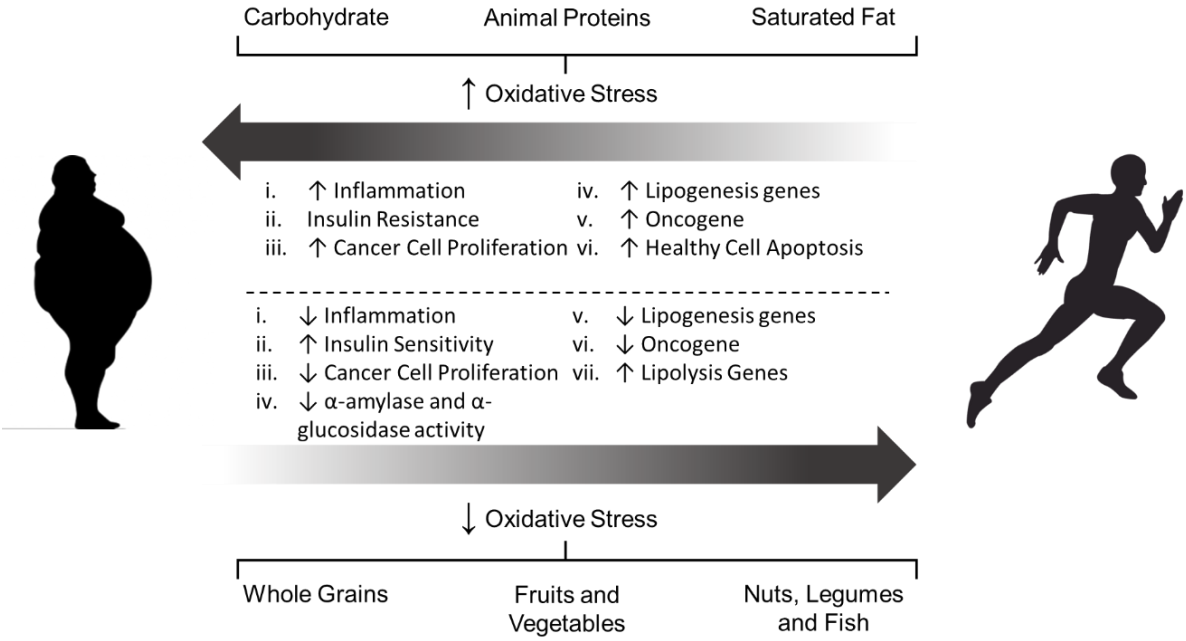
The evidence presented within the literature generally focuses on the consumption of antioxidant supplementation to explore a specific physiological response associated with oxidative stress. However, the role of food consumption with regards to type, and quantity, is still equivocal; thus, a better understanding of the role of dietary choices in relation to oxidative stress and associated physiological conditions is needed.

It is evident diet plays a major role in oxidative stress and associated pathways with diets high in carbohydrate and animal proteins reflecting a higher state of oxidative stress (Guo *et al.*, 2014). This may explain the recent popularity with the Mediterranean diet, Paleo, and or vegan/vegetarianism, which focus on predominantly plant-based food produce (Casas *et al.*, 2014). The inclusion of whole grains in the diet are known to exhibit health-promoting properties such as regulation of gut microbiota (Tachon *et al.*, 2013), inflammation (Detopoulou *et al.*, 2010) and oxidative stress (De Morais Cardoso *et al.*, 2017). This has been demonstrated in relatively recent meta-analysis and indeed the HELGA project; whereby a high whole grain consumption was associated with a reduction in markers associated with cancer risk (Kyro *et al.*, 2013; Aune *et al.*, 2016). These effects could be due to the high concentrations of vitamins, minerals, fibre, phytochemicals and polyphenol compounds within whole grains (Detopoulou *et al.*, 2010; Donkor *et al.*, 2012); all of which have been associated with a reduction in oxidative stress. These effects have been summarised in Figure 2.22.

A significant body of the research has focused on the role and functionality of fruit and vegetable intake on indices associated with oxidative stress. These health benefits are accredited to the high concentration of antioxidant vitamins, minerals, fibre and polyphenolic flavonoids associated with a high dietary intake of fruit and vegetables (Leenders *et al.*, 2014; Saad *et al.*, 2017). These properties have been reported to have positive effects on lipid metabolism, systemic inflammation, vascular

endothelial function, and oxidative stress (Farras *et al.*, 2016; Zhao *et al.*, 2017). There appears to be insufficient consensus within the literature regarding antioxidant intake associated with dietary sources and oxidative stress; the literature appears to be further sporadic within the area of exercise-induced oxidative stress (Trapp *et al.*, 2010). Therefore, further research trials are warranted to evaluate the efficacy of whole-food sources and/or plant-based food products of antioxidants on exercise-induced oxidative stress.

Figure 2.22. An overview of the effects of nutritional patterns, oxidative stress, and the downstream physiological consequences. High-carbohydrate and an animal-based protein diet and excessive fat consumption will eventually lead to obesity as well as other obesity-related diseases. The key pathway involved in the pathogenesis is via the elevation of oxidative stress. Subsequently, inflammation occurs resulting in the reduction of insulin sensitivity, increased cancer cell proliferation, involvement of gene in lipogenesis, and cancer development of which is activated and accompanied by apoptosis of healthy cells. To revert these unhealthy conditions, consumption of healthy diet is essential. Healthy diet includes whole grains, nuts, fruits and vegetables, fish, and legumes. In general, a healthy diet contains dietary fiber, unsaturated fatty acids like monounsaturated fatty acid and n-3 polyunsaturated fatty acid, protein, vitamins, minerals, and others health-promoting components. All these components exhibit antioxidant ability thereby reduce oxidative stress. The healthy diet could reduce inflammation, cancer development, and lipogenesis transcriptional expression. It also increases insulin sensitivity accompanied by the reduction of α-amylase and α-glucosidase activity. A healthy dietary pattern is crucial for maintaining good health.



2.4.5 Exploring the Nutritional Profile of Barley and Wheat Grass

The idea of using grass as food with benefits of health is not new; barley (*Hordeum vulgare L.*) has been cultivated from the Stone Age and is the fourth most important cereal crop in the world (Zeng *et al*., 2018). Similarly, wheat grass (*Triticum aestivum*) is one of the most common cereal grasses used within functional products such as smoothies, juices, and powders; as a result, it is often referred to as '*the new age espresso*' (Ben et al., 2002). Barley and wheat grass are rich sources of nutrients as well as bioactive compounds including proximate parameters, minerals, vitamins, and phenolic compounds; the properties of these grasses will be explored in the forthcoming section.

Barley grass is not only consumed commercially (Ikeguchi *et al.,* 2014), but also used in preventive chronic diseases, especially, circulatory disorders, anti-cancer, antioxidant, and anti-inflammation (Lahouar *et al.,* 2015). Barley grass is rich in nutritious and functional compounds such as vitamin A (20.5 mg/100 g), ascorbic acid (251.6 mg/100 g), chlorophyll (528.5 mg/100 g), SOD (440.0 U/g), and catalase (839 U/g) (Duan *et al.,* 2014; Lahouar *et al.,* 2015; Jiazhen *et al.,* 2016); however, concentration of these bioactive molecules can vary depending on horticultural and botanical conditions, including: growth stage, light sources, temperature, humidity, and soil conditions (Jia *et al.,* 2010; Koga *et al.,* 2013). Due to the abundant nutritional properties of barley grass, it processes a number of health promoting effects such as antidiabetic properties (Lyer & Venugopal, 2010; Yu *et al.*, 2012), blood pressure regulation (Richter *et al.*, 2010; Lahouar *et al.*, 2015), enhanced immunity (Kim *et al.*, 2017), and anti-inflammatory effects (Ferrone *et al.*, 2007; Seo *et al.*, 2014). In addition, the plethora of antioxidants within barley grass (including, *γ*-tocopherol, glutathione, succinate, superoxide dismutase, 2″-*O*-glycosyl isovitexin, protoheme, lutonarin, saponarin, isoorientin, and orientin (Kitta *et al.*, 1991; Osawa *et al.*, 1992; Lee *et al.*, 1994; Choe *et al.*, 2010; Templer *et al.*, 2017), provides precedent for the exploration of a prophylactic effect (if any), against exercise-induced oxidative stress.

Wheat grass contains minerals and trace elements including ascorbic acid, α-tocopherol, and SOD which can attenuate the accumulation of RONS (Kulkami *et al.*, 2006). Similarly, to barley grass, there are a myriad of potential health benefits associated with wheatgrass consumption including hepatoprotective roles, anti-hyperlipidemic, hypoglycemic effect, and anti-inflammatory (Kothari *et al.*, 2008; Grunewald, 2009; Arya & Kumar, 2011). Furthermore, Sethi *et al.* (2010) observed a

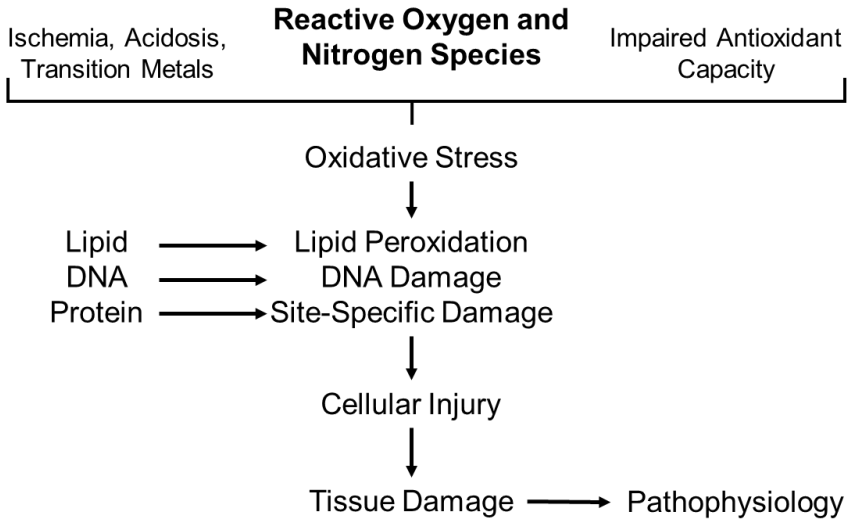
reduction in lipid peroxidation, and a restoration of enzymatic antioxidant concentration following wheatgrass supplementation. In addition, wheatgrass supplementation has also been shown to enhance the antioxidant defence system and simultaneously improve blood sugar of diabetic rats (Shakya *et al.*, 2012; Mis *et al.*, 2018).

Both barley and wheat grass are nutritionally very similar, with barley grass containing marginally more calcium, potassium, chlorophyll, and enzymes than wheatgrass (Rana *et al.,* 2011). Qamar *et al.* (2018) compared the antioxidant profile of barley and wheat grass; ultimately, they determined that barley grass has a higher antioxidant potential as compared to wheat grass. Nevertheless, these grasses are highly dense sources of nutrients and natural antioxidants which may find applications in the formulation of novel functional products could be used against various maladies.

2.5 Biological Consequences of Free Radical Formation

It is clear that RONS are critical for normal human physiology, especially as they are involved in signal transduction (Apel and Hirt, 2004), cell apoptosis (Dangl & Jones, 2001), regulation of the immune system (Apel and Hirt, 2004), growth and development (Gapper & Dolan, 2006), stress acclimation (Gechev *et al.*, 2006), and exercise adaptation (Scheele *et al.*, 2009). On the contrary, chronic and/or high concentrations of RONS are deleterious and injurious, with RONS-driven inflammation being a central core of pathophysiological disease progression (Mittal *et al.*, 2014). To elaborate, although the development of cancer is multifaceted, it is known that oxidative DNA damage is involved in cancer development along with chromosomal instability, upregulation in oncogenes, mutations, and abnormal transcription expression profiles (Valko *et al.*, 2006; Valko *et al.*, 2007). The deleterious nature of RONS and oxidative stress is shared amongst a multitude of pathological conditions including cardiovascular disease (Ceriello, 2008), Alzheimer's and Parkinson's disease (Halliwell, 2001), chronic obstructive pulmonary disease (Guo & Ward, 2007), and rheumatoid arthritis (Mahajan & Tandon, 2004). The mechanistic overview is shown in Figure 2.23 below:

Figure 2.23. A schematic of the mechanisms associated with RONS-mediated disease etiology.



The following section will detail the biological consequences of a free radical insult to these physiological macromolecules and downstream responses. It is also important to highlight that the extent of the damage to these biochemical targets depend on a multitude of factors including; (i) the concentration of the macromolecule, (ii) the rate constant for the specific oxidising agent, (iii) the cellular location of the target substrate in comparison to the site of oxidant generation, (iv) the probability of secondary damaging events and transfer reactions, and, (v) the repair/scavenging abilities of the cell and/or antioxidant system (Davies, 2012).

2.5.1 DNA Damage

DNA is a remarkably unstable molecule, undergoing substantial spontaneous modifications on a regular basis (Lindahl, 1993); even in the absence of any exogenous DNA damaging agents. DNA base lesions can occur as part of normal physiological conditions and estimated to be as high as 20,000 per day; these lesions are generated through hydrolysis, oxidation, and non-enzymatic methylation reactions (Markkanen, 2017). If DNA is left unrepaired, cell dysfunction and oncogenic modifications can occur which may be passed onto daughter cells; as a result, it is integral to cellular and organismal homeostasis that genomic integrity is maintained (Hasty, 2005). Thus, not only is genetic integrity vulnerable during times of pathophysiological stress, but the inherent nature of normal physiology poses a continual challenge to the integrity of DNA. The most common types of endogenous DNA lesions are presented in Table 2.6.

Table 2.6. An overview of the major endogenous DNA lesions. Abbreviations - FaPy-G: 2,6-diamino-4-hydroxy-5-formamidopyrimidine; DSB: double strand break. Adopted from Abbots and Wilson (2017).

DNA Damage	Lesion	Consequences	Rate (~cell/day)	Primary Repair Enzyme
Depurination/ pyrimidation	Abasic site	Mutagenic bypass, replication fork stalling, conversion to DSB	10,000	Apurinic/apyrimidinic endonuclease 1
Cytosine deamination	Uracil	Mutagenic base mispairing	400	Uracil-DNA glycosylase, single strand selective monofunctional uracil DNA glycosylase 1, thymine-DNA glycosylase
5-methylcytocine deamination	Thymine	Mutagenic base mispairing	30	Thymine-DNA glycosylase, methyl-CpG-binding domain protein 4
Methylation	7-methylguanine	Tolerance, abasic site formation, ring-opening to FaPy-G	4,000	N-methylpurine-DNA glycosylase
	5-methyladenine	Replication stalling, chromosomal instability	600	N-methylpurine-DNA glycosylase
Nitrosamine alkylation	O^6-methylguanine	Mutagenic base mispairing	200	O^6-methylguanine-DNA methyltransferase
Reactive species attack, abortive TOP1 etc	Single strand break	Replication fork collapse, conversion to DSB	10,000	Single strand break repair
Base oxidation	8-oxo-g	Mutagenic base mispairing	1,000	Oxoguanine glycosylase; 8- oxo-G: 8-oxo-7,8-dihydroguanosine
Ring saturated pyrimidine	Thymine glycol	Mutagenic base mispairing	500	Endonuclease III homolog 1

2.5.1.1 ROS and DNA Damage

It is now clear that the hydroxyl radical is the primary driver of DNA damage (Tsunoda *et al.*, 2010). The damaging nature of this radical, and high reactivity means it is almost impossible for antioxidants to scavenge it effectively (Forman *et al.*, 2015). The most common reaction associated with DNA and the hydroxyl radical is via the 5,6-pyrimidine and 7,8-purine double bonds of nucleobases (von Sonntag, 1987); secondary reactions include the hydrogen abstraction of thymine and 5-methylcytosine in turn, generating corresponding radicals (Cadet *et al.*, 2010; Wagner & Cadet, 2010). It should be noted that the hydroxyl radical can also attack the methine carbon positions, resulting in the formation of carbon-centred radicals; thus, giving rise to peroxyl radicals and the formation of strand breakage (Cadet *et al.*, 2017).

Peroxyl radicals are conceived from an initial hydroxyl radical-mediated reaction, and they can react slowly with DNA due to their weak oxidant properties. Specifically, in a sequence-dependent manner, they react with proximal guanine bases on the 5' prime end (Bourdat *et al.*, 2000; Douki *et al.*, 2002; Dupont *et al.*, 2013); one of the initial reports of this, detailed the generation of tandem lesions, specifically formylamine and 8-oxo-7,8-dihydroguanine, following thymine-mediated peroxyl interaction with vicinal guanine (Douki *et al.*, 2002). These tandem lesions spearheaded by thymine-mediated peroxyl attack has been confirmed by others (Kanvah *et al.*, 2010). It is important to note at this stage, these pyrimidine peroxyl radicals, and subsequent tandem oxidative end products are based on cell models, and to the authors knowledge the evidence in mammalian cell DNA is limited (Cadet *et al.*, 2017).

2.5.1.2 DNA Base Oxidation

In addition to DNA damage as a result of the hydroxyl radical and peroxynitrite, singlet oxygen may also have the potential to indirectly damage guanine (Cadet *et al.*, 2010). Superoxide and hydrogen peroxide are almost totally unreactive with DNA bases; however, in the presence of transition metal ions, hydrogen peroxide can undergo Fenton-driven univalent reduction producing the hydroxyl radical. Generally, DNA base damage by the hydroxyl radical reacts at diffusion-controlled rates (~3-10 x 10^9 M^{-1} s^{-1}); the only exception to this, is the methyl group of thymine, and the 2-amino group of guanine as these reactions are driven by competitive hydrogen abstraction (~2 x 10^9 M^{-1} s^{-1}) (Cadet *et al.*, 1997; Chatgilialoglu *et al.*, 2009).

With respect to cytosine and thymine, hydroxyl reactions to the C5 and C6 positions will generally form a corresponding DNA-adduct radical; subsequently, these can react with 5-hydroxy-6-peroxyl radicals and superoxide leading to the downstream formation of cytosine glycol and thymine glycol respectively (Dizdaroglu, 1992; Breen & Murphy, 1995). On the other hand, the hydroxyl radical reacts slightly different with the larger purines; adenine and guanine. Oxidation of the C4 and C5 positions result in the generation of purine radicals, however there is also potential for the oxidation and/or reduction of the C8 position leading to the generation of 7,8-dihydro8-oxopurines and formamidopyrimidines; for example, 7,8-dihydro-8-oxo-2′-deoxy-Guanine (8-oxo-G) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) (Dizdaroglu, 1998; Steenken, 1989).

Of all the base lesions presented above, the most common RONS-driven DNA lesion is typically the 8-oxo-G modification; owing to the low reduction potential of guanine (1.29 V) (van Loon *et al.*, 2010). The conception of this lesion rises from the addition of an oxo-group at C8 and hydrogen at N7. It has been reported this type of lesion occurs at approximately 10^3 times per cell per day, with these figures being compounded in diseased states/cells (~10^5). The main consequential factor of 8-oxo-G aside from lower reduction potential of guanine (0.74 V), is its ability to mimic thymine (Steenken *et al.*, 2000); particularly when in the *syn* conformation, as this can lead to the pro-mutagenic mispair (adenine:8-oxo-G), and consequentially a C:G → A:T transversion mutation (Markkanen, 2017).

In addition to the reactions outlined above, the hydroxyl radical may also abstract a hydrogen from the carbons of the DNA sugar moiety, further propagating the generation of sugar adducts, strand breaks and base-free sites (Sonntag, 1987). These potentially can be released or bound from DNA via phosphate linkage. As a general characteristic, abstraction of a hydrogen atom from the sugar-phosphate backbone via competitive steric accessibility, causes the formation of 2-deoxyribose radicals which in turn can lead to strand damage (Gates, 2009).

2.5.1.3 DNA Sugar Damage

At a rate of ~2 x 10^9 M^{-1} s^{-1}, the hydroxyl radical abstracts a hydrogen atom from each of the carbon sugar moieties, specifically on the 2'-deoxyribose, leading to the subsequent generation of carbon-based radicals; in turn, these can yield further damage. It has been hypothesised the extent of this reaction equates to approximately

20%, with the carbon atom availability acting as the bottleneck of the reaction (von Sonntag, 2006); additionally, this reaction also appears to differ within the nucleus as determined by the extent of OH· attack within poly(U) is approximately 7% (Deeble *et al.*, 1986). Depending on the nature of the resulting products, it has been suggested certain end products can lead to the formation of DNA strand breakage and abasic sites (Dizdaroglu *et al.*, 1975; Dizdaroglu, 1998). Furthermore, particular free modified sugars may be released from DNA; on the contrary, others remain within DNA and encompass broken end groups of DNA strand damage (Evans *et al.*, 2004). To expound upon, one such reaction of the hydroxyl radical is the formation of the alkoxyalkyl C4-radical with having the defining characteristic of a phosphate group in the β-position. This conformation provides the flexibility of the phosphate to be released on either side of the DNA chain; lending to the radicals' ability to consequently generate strand breaks and end group cation radicals (Behrens *et al.*, 1982). It appears that the generation and subsequent reactions of the C4-radical will lead to DNA single strand breaks in the absence of oxygen as detailed by Dizdaroglu *et al.*, (1975). This is due to the heterolytic phosphate-cleavage at C3' and C5'; consequently, the addition of HO· to the generated radical cations is followed by reduction reactions, resulting in DNA strand breakage (Dizdaroglu & Jaruga, 2012).

2.5.1.4 DNA Double Stranded Damage

One of the least frequent types of DNA damage are double stranded breaks (DSBs) and are formed as a result of the simultaneous destruction to the two complementary DNA phosphate backbones (Jeggo & Mobrich, 2007). In spite of the infrequency of DNA DSBs, these are one of, if not *the* most, toxic lesions produced within DNA, owing to the fact one DNA DSB can induce chromosomal rearrangements and genomic instability (Paulovich *et al.*, 1997). Generally, this type of damage is conceived from the use of exogenous pharmacological drugs (especially those of a chemotherapeutic nature) and ionizing radiation (Cannan & Pederson, 2017). DNA DSBs tend to be associated with exogenous sources, and there are a variety of endogenous physiological processes that can induce these genotoxic lesions, including DNA replication, inadvertent action of nuclear enzymes, mechanical stress on the DNA duplex, and intentional programmed induction of DSBs (de Massey, 2013).

The most common mechanism of DSB's is through the endogenous process of DNA replication as outlined by Syeda and colleagues (2014). Upon replication, the

presence of a single-strand break can lead to a replication fork arrest and subsequent induction of DSBs (Pfeiffer *et al.*, 2000). It should be noted that the presence of the single strand break may be a repair intermediate, however other unusual structures such as bulky lesions, crosslinks, abasic sites and certain binding proteins can all include a DSB during replication-associated processes (Prado & Aguilera, 2005; Dextraze *et al.*, 2010).

Moreover, DNA DSBs can occur from gapped repair intermediates during the base excision repair pathway when attempting to rectify clustered lesions (David *et al.*, 2007, Duclos *et al.*, 2012). Generally during base excision repair, DNA polymerase β and DNA ligase IIIα attempts to seal the 1-2 nucleotide gap; however, in the instance of clustered lesions, the inefficient repair of single strand breaks on parallel strands can lead to the conversion of DSBs (Eccles *et al.*, 2011). It should be noted, that not only is there compelling evidence that base excision repair (BER)-driven DSB formation occurs *in vitro* (Sage & Harrison, 2011), but there is also substantial evidence *in vivo* (Blaisdell & Wallace, 2001; Yang *et al.*, 2006); demonstrating that knockout of DNA glycosylases reduced the frequency (and vice-versa); indicating BER has a role in the indirect formation of DNA DSBs.

2.5.2 DNA Repair

There are over 100 known oxidative lesions and 2-deoxyribose modifications (Cadet *et al.*, 2010), that can induce deleterious effects to DNA replication and transcription process; it is these consequential mutations and chromosomal aberrations that contribute to the progression of pathological conditions associated with oxidative DNA damage (Polo & Jackson, 2011). As a result, cells have evolved with an elaborate DNA-damage repair response network. Although the pathways associated with the DNA-damage repair responses are typically lesion-specific, similarities are shared across a number of these processes (Giglia-Mari *et al.*, 2011); these are summarised in Table 2.7. and Table 2.8.

Table 2.7. General classification of the major steps associated with DNA repair and corresponding DNA repair proteins.

Phases of DNA Repair	Corresponding Repair Proteins
Sensing	Glycosylases, Helicases
Reinforcement	Scaffolders, Stablisers
Excision	Endonucleases, Lyases
Reconstruction	Polymerases, Progressivity factors
Restoration	Exonucleases, Ligases

2.5.2.1 Direct Repair

The direct repair pathway is the only mechanism that can repair DNA damage in a single process without the need to excise bases or disrupt the phosphodiester backbone (Plummer *et al.*, 2010). Direct repair is responsible for the repair of methylated guanine and thymine bases as a result of normal physiological metabolism, and exogenous damaging agents by the removal of an alkyl group (Kaina *et al.*, 2007). The protein complex responsible for this repair pathway is the sacrificial enzyme O^6-methylguanine DNA methyltransferase (MGMT) which is degraded once it stoichiometrically transfers the methyl group from the oxidised base (Fleck & Nielsen, 2004).

2.5.2.2 Base Excision Repair

When DNA damage is minor and induces no significant architectural changes to the double helical structure of DNA (i.e. 8-oxo-dG, thymineglycol, abasic sites, single strand breaks, N^7-Alkyl-dG, N^3-Alkyl-dA etc.), base excision repair (BER) is the typical repair pathway that is recruited (Almeida & Sobol, 2007; Hegde *et al.*, 2008). BER is a highly orchestrated pathway responsible for the repair of many oxidation, alkylation, deamination, and IR reactions associated with DNA damage (Luo *et al.*, 2010); depending on the extent of damage, one of the two sub-categories of BER will be recruited. Short-Patch BER will typically only response to single-base damage, in contrast to Long Patch BER which has the ability to repair 2-8 oxidised/reduced nucleotide sequences; although a slightly longer process which requires additional repair proteins, cells will switch between the two sub-pathways depending on substrate availability (Robertson *et al.*, 2009).

The initial DNA damage-repair response consists of the induction of one of 12 lesion-specific DNA glycosylase enzymes (Robertson *et al.*, 2009), which will monitor

and scan the DNA-sugar backbone in an attempt to locate the chemical alteration. These lesions-specific glycosylases work in conjunction with abasic-endonucleases, BER-specific polymerases (β), and ligases (XRCC1/ligase III) to cleave the *N*-glycosidic bond and remove the damaged base, fill the nucleotide gap, and incorporate the new base, respectively (Giglia-Mari *et al.*, 2011). Due to the prevalence of this pathway, a safety mechanism exists whereby glycosylases remain bound to the damaged site until the next chronological enzyme binds (Vidal *et al.*, 2001); this ability allows the damaging intermediates to be chaperoned until repair has been complete (Hegde *et al.*, 2010; Williams & Kunkel, 2011).

2.5.2.3 Mismatch Repair

Mismatch repair (MMR) has three primary objectives: (i) it acts to improve fidelity of the replication process (Guarne & Charbonnier, 2015); (ii) MMR is responsible for the post-replicative sensing of single-base mismatches and misaligned nucleotides (Kelley & Fishel, 2016); and finally, (iii) MMR acts to correct errors in the genomic code to reduce the potential frameshift mutations and microsatellite instabilities (Kinsella, 2009). One critical caveat to MMR relates to is ability to only correct damage on the newly formed daughter strand; however, when damage on the parent strand, such as O^6-methylguanine (O^6meG) or 6-thioguanine (6TG), causes mispairing at replication, the MMR attempts to repair the newly synthesized daughter strand, rather than the damaged one, resulting in the increased likelihood of a DSB (Kelley & Fishel, 2008; Curtin, 2012).

2.5.2.4 Nucleotide Excision Repair

The multi-faceted nucleotide excision repair (NER) pathway is typically activated when the damage is across stretches of DNA, in turn creating a local DNA-helix subversion which can interfere with DNA replication and transcription (Kelley & Fischel, 2016). These lesions include, but are not limited to, bulky adducts (i.e. Benzo[α]pyrene, aflatoxins and nitrosamines), intra-strand crosslinks, cyclobutane pyrimidine dimers, 6-4 pyrimidine-pyrimidone photo-products, and although currently polemical, some evidence suggests the repair and/or transformation of 5-Me-C is through a GADD45a-dependent NER process (Barreto *et al.*, 2007). Transcription-coupled NER (TC-NER) allows for the rapid repair of lesions which have the potential to stall transcription, whereas global-genome NER (GG-NER) can repair structural damage anywhere in the

genome (Bohr *et al.*, 1986; Hanawalt, 1994). Upon recognition of DNA damage, Cockayne syndrome factors A and B will assemble the TC-NER repair pathway (Fousteri & Mullenders, 2008), whereas a protein network of XPC/hHR23B and DDB1(2)/XPE will sense damage, thus initiating GG-NER (Sugasawa *et al.*, 2009). Following the initial damage sensing, the repair pathways subsequently converge into a shared repair mechanism of splitting and unwinding the damaged section across the approximate length of 30 nucleotides; this process is achieved through the sequential activation and recruitment of Transcription Factor IIH, XPA, and Replication Protein A (de Laat *et al.*, 1998; Sugasawa *et al.*, 2009). This is followed by incision and removal of the 30-base strand, mediated by endonucleases XPG and ERCC1-XPF; which is succeeded by normal polymerase (δ, ε, or κ) and ligase activity (I/III) (O'Donovan *et al.*, 1994; Sijbers *et al.*, 1996; Moser *et al.*, 2007; Ogi *et al.*, 2010).

2.5.2.5 Double Strand Break Repair

As stated, the greatest toxicity to DNA is double-strand breaks, as not only do they have the ability to cause mutations, but if unrepaired they can induce translocations and genome amplifications (Underhill *et al.*, 2011; Kamal *et al.*, 2015). Double strand breaks are notoriously difficult to repair due to the variation in broken ends and the general loss of information/stability on body strands of the DNA (Lieber *et al.*, 2003). The repair of double stranded damage is primarily mediated through the use of checkpoint proteins which examine a plethora of damage characteristics, such as the progression of cell cycle phases, the dissemination of the type of break (nonligatable vs. 'blunt'), and the complexity of repair required for said damage (Leiber *et al.*, 2003; Helleday *et al.*, 2007).

The initial response to double-stranded DNA damage is the recruitment of the repair sensors MRE11, RAD50 and NSB1; collectively referred to as the MRN complex (Lafrance-Vanasse *et al.*, 2015). This assesses the appropriate sub-category of repair which should be mobilised. Following MRN activation, a number of checkpoint proteins will be activated; specifically, ataxia telangiectasia mutated and related-3 (ATM/ATR), and DNA-dependent phosphokinases (DNA-PKcs) (Falck *et al.*, 2005). This subsequently signals for one of the earliest DNA double stranded damage responses; phosphorylation of the histone H2A-variant H2AX. Detection and observation of increasing amounts of *γ*-H2AX foci is generally associated with increasing amounts of

DNA DSBs suggesting they occupy a dominant role in the DNA damage repair response (Giglia-Mari *et al.*, 2011). Although the correlation between the number of *γ*-H2AX foci, and the exact number of DSBs, remains controversial (Lobrich *et al.*, 2010), it is well accepted that *γ*-H2AX detection characterises a DNA DSB; while disappearance of *γ*-H2AX foci characterises repair of this damage (Mariotti *et al.*, 2013)

2.5.2.5.1 Non-Homologous End Joining

Non-Homologous End Joining (NHEJ) is the preferred pathway due to its rapid, simplistic response, and can execute repair during all stages of the cell cycle; the only impediments to this pathway, are that it is susceptible to errors, and nonligatable breaks often require the loss of genetic material (Curtin, 2012; Kakarougkas & Jeggo, 2014). NHEJ has 4 key stages to the repair process of double strand breaks; (i) detection and protection, (ii) removal of unligatable end groups, (iii) replacements/reinstatement, and (iv) sealing. Many aspects of NHEJ still remain unidentified; for example, how the pathway recruits the necessary, specific repair proteins (Davis & Chen, 2014), or the ability of NHEJ-related polymerases to unconventially 'proof read' and detect gaps in the 'repaired' strand (Ramsden & Asagoshi, 2012).

2.5.2.5.2 Homologous Recombination Repair

There are two distinct features that separate the primary pathways of DNA DSB repair; firstly, homologous recombination repair (HRR) is template-mediated through the sister chromatid, homologous chromosomes, or repeated regions on the same/different chromosomes. Secondly, as a result, HRR only occurs post-replication during phases S and G2 of the cell cycle; as a full copy of the DNA is proximally available (Kelley & Fishel, 2016). Subsequently, the complexity of HRR requires the use of a template and thus, the repair process takes longer in comparison to NHEJ; however, the remuneration for this, is a more meticulous and accurate repair of the DNA DSB (Helleday, 2010; Curtin, 2012; Deriano & Roth, 2013).

2.5.2.5.3 DNA Double Strand Repair: Choosing the Right Path

At this stage, it is clear that repair of highly genotoxic DNA double stranded damage is not without admonition. NHEJ exists as a rapid response to DNA DSB damage and operates in all stages of the cell cycle; however, NHEJ is associated with short deletions and base errors, increasing the susceptibility to mutations (Difilippantonio *et al.*, 2000). However, to be critical of this, it is important to highlight that the repair proteins associated with NHEJ play significant roles in genomic stability and repressing tumorigenesis (Karanjawala, 1999; Tong *et al.*, 2002; Zha *et al.*, 2007). Alternatively, HRR possesses a high degree of fidelity, yet it requires specific, error-prone polymerases and is confined to specific phases of the cell cycle (Malkova & Haber, 2012). Similarly, the active roles of HRR proteins in genomic stability and suppressing tumorigenesis are well documented (Nickoloff, 2002). Cells appear to choose NHEJ as their preferential repair mechanism; however, failing to do so, one strand of the DNA double strand break will become resectioned to form an overhanging end allowing for alternative repair mechanisms (Rothkamm *et al.*, 2003; Beucher *et al.*, 2009; Kowalczykowski, 2015). Additionally, a number of associated repair proteins appear to dictate the choice between pathways.

One such accessory factor, is the dominance of 53BP1 in contrast to other DNA repair factors, such as BRCA1. 53BP1 is a large binding protein for the tumour suppressor protein, p53 (Iwabuchi *et al.*, 1994). One of its key functioning roles is to inhibit double strand break end resectioning, and to further promote NHEJ. Upon double stranded DNA damage, 53BP1 and BRCA1 co-exist in an antagonist manner, and accumulate at very specific sites of the initial break (Chapman *et al.*, 2012a). It appears that when the cell is within the S and G2 phases, BRCA1 will interact with downstream targets of 53BP1; specifically, BRCA1 will inhibit and subsequently polyubiquitinate RAP1-interacting factor, thus antagonises 53BP1-RIF1 in S–G2 in favour of homologous recombination (Li & Xu, 2016). Additionally, there is evidence to suggest that BRCA1 mediates the foci formation of HRR proteins, namely RAD51 (Prakash *et al.*, 2015). The exact mechanism of how BRCA1 can contravene the effects of 53BP1 within these specific phases of the cell cycle remains enigmatic (Chapman *et al.*, 2012b).

Table 2.8 Overview of the major DNA repair pathways and associated lesions and repair proteins.

DNA Repair Pathways	Primary Corresponding Lesions	DNA Repair Proteins
Direct Repair	O6-alkylguanine	MGMT
Base Excision Repair	Simple base adducts, intermediates from single strand breaks and/or abortive topoisomerase I activity	DNA glycosylases, APE1 endonuclease, DNA polymerases, DNA ligase I/III, XRCC1, PARP-1/2
Mismatch Repair	DNA mismatches and insertion/deletion loops	MSH2:MSG6/3, Exo1, DNA polymerases δ and ε, PCNA, RFC, RPA, DNA ligase I
Nucleotide Repair	Larger architectural changes to the double-helix (bulky lesions)	RNA polymerase, XPA, XPE, XPF/ERCC1, DNA polymerases, RPA, DNA ligase I
Non-Homologous End Joining	Double strand breaks, V(D)J and CSR intermediates	Ku, DNA-PK, XRCC4, DNA ligase IV, MRE11, RAD50, NBS1, DNA polymerase
Homologous Recombination	Double strand breaks, intra-strand crosslinks	RAD51, RAD52, RAD54, XRCC2/3 BRCA2, RPA, DNA polymerase

Abbreviations: MGMT - O^6-methylguanine DNA methyltransferase; APE1 - Apurinic/apyrimidinic endonuclease; XRCC1 - X-Ray Repair Cross Complementing 1; PARP - poly-ADP ribose polymerase; MSH - MutS protein homolog; Exo1 – Exonuclease 1; PCNA - Proliferating Cell Nuclear Antigen; RFC – Replication Factor C; RPA – Replication Protein A; XPA - Xeroderma Pigmentosum Group A-Complementing Protein; XPE - Xeroderma Pigmentosum Complementation Group E Protein; XPF/ERCC1 - ERCC Excision Repair 1; DNA-PH – DNA-Dependent Protein Kinase; MRE11 - Meiotic Recombination 11; NBS1 – Nibrin; BRCA - Breast Cancer Susceptibility Gene.

2.5.3 Ribonucleic Acid Oxidation

Arguably, until the past decade or so, free radical and redox biologists (particularly in the context of exercise) have overlooked the role of ribonucleic acid (RNA) in oxidative biochemistry. From a molecular perspective, there is a substantial body of evidence which suggests RNA is more vulnerable to oxidative damage in contrast to other biological substrates (Kong & Lin, 2010); this susceptibility can potentially be clarified through a number of physiological factors. Firstly, on a weight-by-weight basis, RNA is approximately 4 times greater than DNA in mammalian cells (Fimognari, 2015). Secondly, RNA bases are not protected by hydrogen bonds, and certain RNA classes have a high affinity for iron; in turn, promoting the potentiation of Haber-Weiss and/or

Fenton chemistry (Honda *et al.*, 2005; Wurtmann & Wolin, 2009). Moreover, RNA is single stranded and has a lower association with proteins, thus making it more accessible to oxidative attack (Li *et al.*, 2006). Finally, the repair capacity of RNA is still not fully understood nor to what extent oxidatively damaged RNA is removed (Hofer *et al.*, 2008). These factors explain RNAs vulnerability to oxidative attack, and it has been estimated that RNA oxidation is 10-20 times greater than DNA oxidation (Li *et al.*, 2014).

The mechanism of oxidative damage to RNA is primarily through the hydroxyl radical; resulting in the formation of over 20 identified end products (Kong & Lin, 2010), with the deleterious 8-hydroxyguanosine (8-OHG) modification being the most protuberant. 8-OHG can lead to mispairing with adenine or thymine resulting in the mis-incorporation of nucleotides during DNA/RNA synthesis (Taddei *et al.*, 1997; Hofer *et al.*, 2006). Additionally, similar to DNA, oxidative damage to RNA can take the form of ribose modifications, base alterations, and strand breakage (Fimognari, 2015). It should be noted, the evidence surrounding exercise-induced RNA oxidation is lacking and further research is warranted.

2.5.4 Lipid Peroxidation

Lipids are classically split into categorial divisions of *apolar* and *polar*; the former primarily being used for energy storage, with the latter acting as a structural component within cell membranes (Fruhbeck *et al.*, 2001). This ability of polar lipids to act as structural molecules lends well to the signalling properties of lipid membranes. Modifications to the biophysical state of the lipid membrane in the context of polarity and permeability changes, exerts control over the physiology state of membrane organelles (Ayala *et al.*, 2014).

Lipid peroxidation is one of the most commonly cited consequences of oxidative damage (Halliwell & Chirico, 1993) and is characterised as a propagating chain reaction. Similarly, to DNA damage, lipid peroxidation is largely driven by hydroxyl radical attack; however, a second destructive radical, the hydroperoxyl radical ($HO\cdot_2$), also has a profound effect on lipid integrity via oxidation of polyunsaturated phospholipids (Bielski *et al.*, 1983; Schneider *et al.*, 2008). It has been suggested that approximately 4 million hydroxyl radicals are produced per day in each cell, which indiscriminately attack biological structures within nanometres of their generation (Halliwell & Gutteridge, 1984; Lane, 2002). The insult of the hydroxyl radical is

unspecified, with the attack on lipids being no different; phospholipids, glycolipids and cholesterol are all prime targets of peroxidative modification (Ayala *et al.*, 2014). Research indicates that lipid peroxidation may play a role in the development of several pathological conditions such as atherosclerotic lesions (Steinberg *et al.*, 1989), Parkinson's disease (Farooqui & Farooqui, 2011), Alzheimer's disease (Famulari *et al.*, 1996), gastric diseases (Repetto *et al.*, 2003), and, other neurodegenerative and nutritional conditions (Dominguez *et al.*, 2008; Repetto *et al.*, 2010).

Lipid peroxidation is a relatively complex series of reactions; however, at its simplest form, it characterises the repetitive hydrogen abstraction and insertion of oxygen within lipids containing carbon-carbon double bonds, specifically polyunsaturated fatty acids (due to their activated methylene bridge). This results in the generation of lipid-derived peroxyl radicals and hydroperoxides (Yin *et al.*, 2011). Under physiological circumstances, lipid peroxidation of cellular membranes is largely controlled by endogenous antioxidants and signalling pathways which mitigate the detrimental effects associated with lipid peroxidation and upregulate an adaptive response. On the other hand, during toxic states associated with lipid peroxidation (such as oxidative stress), there is substantial macromolecule damage, leading to an inability of repair; subsequently, contributing to the potential for cellular apoptosis and necrosis (Reis & Spickett, 2012; Volinsky & Kinnunen, 2013).

The process of lipid peroxidation can be divided into 3 distinct, interlinking phases; initiation of the chain reaction, propagation, and finally, termination (Kanner *et al.*, 1987). The initiation step involves the abstraction of the allylic hydrogen, and sequential generation of a carbon-centred lipid radical (L·). This newly formed radical is relatively stable as it changes its molecular rearrangement of double bonds upon generation, to form a conjugated diene. This then interacts with oxygen to generate the lipid peroxyl radical (LOO·) and subsequently abstracts another allylic hydrogen from the adjacent lipid; in turn, producing a new lipid radical and lipid-derived hydroperoxide (LOOH); this two-step process encompasses the propagation phase, implying a single initial abstraction, can generate multiple hydroperoxides. This phase will continue to potentiate until the final termination phase. This concluding step to the lipid peroxidative process is summarised by the antioxidant-mediated hydrogen donation (α-tocopherol, for example) to the lipid peroxyl radical, in turn producing an antioxidant-radical and further reaction with another peroxyl radical; as a result, a

nonradical derived from the LOO· remains (Yin *et al.*, 2011). A summary of these reactions is detailed below:

(Initiation)	$R1H + R\cdot \rightarrow R1\cdot + RH$
	$OH\cdot + RH \rightarrow R\cdot + H_2O$
(Propagation)	$R\cdot + O_2 \rightarrow ROO$
	$R1H + ROO\cdot \rightarrow R1\cdot + ROOH$
(Termination)	$ROO\cdot + ROO\cdot \rightarrow ROH + RO\cdot + {}^1O_2$
(Transition Metal Re-Initiation)	$ROOH + Me^{n+} \rightarrow RO\cdot + Me^{(n-1)+}$
	$ROOH + Me^{(n-1)+} \rightarrow ROO\cdot + Me^{n+}$

Although the primary products of lipid peroxidation are lipid hydroperoxides, there are several other oxidative-derived products generated. For example, secondary aldehydes such as malondialdehyde (MDA), hexanal, and 4-hydroxynonenal (4-HNE) have been used to characterise the mechanisms and dynamics associated with lipid peroxidation.

With respect to secondary products of lipid peroxidation, historically MDA has been used as a biomarker of the oxidation of omega-3 and omega-6 fatty acids using the thiobarbituric acid reacting substances (TBARS) assay (Esterbauer & Cheeseman, 1990). This fluorometric-based assay works on the premise that MDA reacts with thiobarbituric acid; however, as recently highlighted by Cobley *et al.* (2017), due to the notorious lack of specificity (at 532 nm), and artefactual MDA, the TBARS assay has since been abandoned. Quantification of MDA has now been replaced with more accurate, liquid- and gas-chromatography mass spectrometry-based approaches (Giera *et al.*, 2012). Nevertheless, MDA is still one of the most popular markers of oxidative stress within the clinical setting (Giera *et al.*, 2012). Although MDA is typically viewed from a negative perspective due to its ability to generate DNA adducts and cellular/tissular protein damage, there is some evidence which highlights the potential signalling functions of MDA (Garcia-Ruiz *et al.*, 2002; Wang *et al.*, 2014).

2.5.5 Protein Oxidative Damage

A final major target substrate for oxidative attack is intracellular proteins; potentially resulting in the etiology of pathological disease and the aging process (Stadtman & Berlett, 1997; Levine & Stadtman, 2000). The presence of elevated oxidatively

damaged protein derivatives are a common denominator shared across several pathological diseases such as Alzheimer's and Parkinson's disease (Cole *et al.*, 2005; Masters & Selkoe, 2012), cataractogenesis (Garland *et al.*, 1988), arthritis (Chapman *et al.*, 1989), diabetes (Jones & Hothersall, 1993), and Werner's syndrome (Berlett & Stadtman, 1997).

Proteins are liable to oxidative attack due to their prominence within biological cellular systems and the fast rate constant of the oxidants (Davies, 2012). For example, within plasma (~70 g protein dm^{-3}), liver tissue (~140 g kg^{-1} wet mass), and leukocytes (~100 g per 10^{12} cells), proteins are present in abundance, with other components significantly lower (Davies, 2005).

A multitude of oxidative protein end products can be generated depending on the nature and reactivity of the damage; this is primarily driven by the location of the oxidative attack (i.e. the protein backbone and the 20 common amino acid side chains) (Davies, 2012). For instance, the hydroxyl radical has a positional selectivity for the amine group on the α-carbon of free amino acids (Kopoldova *et al.*, 1963; Hawkins & Davies, 1998); this is accompanied by reactivity variation depending on the specific amino acid (e.g. 1×10^7 M^{-1} s^{-1} for glycine, in contrast to 1×10^{10} M^{-1} s^{-1} for tryptophan, histidine, and cysteine; Buxton *et al*, 1988). It should also be noted that specific amino acids react rapidly, and preferentially, such as addition reactions (phenylalanine, tyrosine, tryptophan and histidine), in response to transitory energy states, and abstraction reactions due to the presence of thiol groups (cystine) (von Sonntag, 1987; Armstrong, 1990; von Sonntag, 1990). This selectivity of oxidative attack can result in the formation of amino-specific oxidation products including nitrotryptophan and kynurenine (tryptophan); 2,3-Dihydroxyphenylalanine and 4-hydroxyphenylalanine (phenylalanine); 3,4-Dihydroxyphenylalanine and tyrosine–tyrosine cross-linkages (tyrosine); glutamic semialdehyde (arginine); a-Aminoadipic semialdehyde (lysine) to name a few (Berlett & Stadtman, 1997; Phaniendra *et al.*, 2014).

Due to the wide-reaching effects of proteins in biological systems, oxidative damage to these central molecules can result in a number of deleterious downstream effects including; impaired functionality, loss of enzymatic activity, reduction in transport proteins, and loss of receptor function (Butterfield *et al.*, 1998). For these reasons, the quantification of oxidative damage to proteins to further elucidate the role of proteomics in physiology and pathology is highly desirable.

Although RONS-mediated protein damage can result in multiple oxidation products, the presence of carbonyl groups and/or derivatives, have been commonly used as biomarkers of oxidative damage (Chevion *et al.*, 2000). With that being said, it is clear that RONS are not the only molecules capable of damaging proteins, and as such, O-tyrosine and 3-nitrotyrosine are often used as specific markers of the hydroxyl- and RNS-mediated damage to proteins, respectively (Phaniendra *et al.*, 2014). Typically, detection and quantification of these oxidative markers can be performed through immunochemical and/or spectrophotometric based assays (Levine *et al.*, 2000; Dalle-Donne *et al.*, 2003; Grimsrud *et al.*, 2008); however, others have used mass spectrometry approaches (Madian & Regnier, 2010; Bollineni *et al.*, 2011).

2.5.6 Redox Basis of Exercise

Redox processes are increasingly recognized as an integral part of the exercise-associated metabolism. For one, Cheng *et al.* (2015) and Andrade *et al.* (1998; 2001) have shown that temporal exposure (<4m min) or low concentration (nM range) of hydrogen peroxide increased muscle force production, while the addition of the antioxidant dithiothreitol resulted in a decrease in force production. On the contrary. prolonged exposure (>8min) or high concentration (mM range) of hydrogen peroxide decreased force production which was attenuated by antioxidant supplementation. It has been suggested that hydrogen peroxide regulates skeletal muscle force production by altering myofibrillar calcium sensitivity. This relationship between exercise-induced RONS and muscle force production (and muscle fatigue) is depicted in the classic biphasic model suggested by Reid (2001). It should also be noted that superoxide and NO· have been suggested to affect muscle contraction by regulating sarcoplasmic reticulum calcium release and myofibrillar calcium sensitivity respectively (Andrade *et al.,* 1998; Burton *et al.,* 2008). Readers are directed to Cheng *et al.* (2016) for a more comprehensive analysis on exercise-induced redox regulation of muscle contractile function.

During exercise, skeletal muscle tissue coordinates a complex metabolic pathway of glucose uptake to meet the increased energy demands. There is increasing evidence to suggest that cytosolic RONS production by NADPH oxidase regulates muscle glucose uptake and GLUT4 translocation during exercise (Henriquez-Olguin *et al.,* 2019). Sandström and colleagues (2006) treated an ex-vivo muscle preparation with the non-specific antioxidant N-acetylcysteine or Ebselen (a glutathione peroxidase

mimetic) and observed that both antioxidant interventions attenuated the increased muscle glucose uptake during contractions (but not resting uptake). Additionally, the authors also examined the effect of N-acetylcysteine on the activity of the redox-regulated AMPK. They concluded a dampened response following antioxidant administration indicating that RONS may regulate glucose uptake via AMPK; it should be noted however, that this relationship has not been fully supported using ex-vivo (Merry *et al.,* 2010a; 2010b; 2010c), or human models (Merry *et al.,* 2010d). Further work is needed, especially for the RONS, to reveal the potential underlying mechanisms involved.

One of initial physiological responses to exercise is the central (e.g. increased cardiac output) and peripheral (e.g. vascular smooth muscle relaxation) changes in hemodynamics in order to match the oxygen and energy substrate demands, and to remove metabolic by-products (Rowell, 2004). It is well established that NO· plays a potent role in vascular function during exercise; especially in the peri-exercise period. In support of this hypothesis, Donato *et al.* (2010). and Richardson *et al.* (2007), observed a decrease in exercise-induced brachial artery vasodilation as a function of antioxidant treatment (ascorbic acid, vitamin E, and alpha lipoic acid); this was in tandem with a decrease in RONS generation as assessed by EPR spectroscopy. The potential role of RONS in vascular function during exercise, along with their interaction (e.g., superoxide reduces the bioavailability of NO·), as well as their synergistic or antagonistic effects with other vasoactive molecules (e.g., acetylcholine) remain unknown, at least from a mechanistic perspective (Hellsten *et al.,* 2012; Margartielis *et al.,* 2020). It seems that RONS have a potent effect on vascular function during exercise, however, their effects on skeletal muscle blood flow remains partially obscure due to the interaction with other local vasoactive molecules (e.g. p38 MAPK, cGMP); readers are directed towards reviews by Trinity *et al. (*2016), and Kadlec and Gutterman (2019) for comprehensive analysis on redox regulation of the vasculature.

More recently there has been an emerging theme of redox bioenergetics, which represents the intriguing interplay between metabolic- and redox-pathways of the cell, and transducing signals that fine-tune physiological responses, such as cell proliferation, differentiation, and apoptosis (Kramer & Darley-Usmar, 2015). One such example, is the mitochondria; a key source, regulator, and target of RONS which can alter mitochondrial, and nuclear and cytosolic pathways (through retrograde signalling) (Cadenas, 2004; Wende *et al.,* 2016). Furthermore, aside from their role in ATP

production, mitochondria are involved in other redox-activated cellular processes either by directly producing RONS or by regulating NAD^+/NADH and $NADP^+$/NADPH homeostasis (Dikalov, 2011). The NAD^+/NADH and $NADP^+$/NADPH couples orchestrate many catabolic and anabolic processes, respectively, to conserve cell survival at the interface between energy metabolism and redox signalling. NAPDH serves as substrate for NADPH oxidases generating RONS, but at the same time it represents the most important reductive substrate for major antioxidant enzymes, such as peroxiredoxins and glutathione peroxidases (Pollak *et al.,* 2007). Collectively, a strong interplay exists between cellular energetic processes and redox reactions, since energy metabolism is both a source and target of RONS. Considering that redox reactions have been linked, at least partially, to some central metabolic molecules strongly associated with exercise metabolism (AMPK, TCA enzymes, succinate dehydrogenase, ATP synthase, creatine kinase, cytochrome c oxidase, GAPDH etc), it becomes evident that the field of redox bioenergetics is one of the most unexplored areas of exercise physiology.

The former section focuses on the acute redox-regulated exercise responses; however, it is important to be aware of the redox-regulated adaptations to exercise training. One such adaptation is an increase in the number and volume density of skeletal muscle mitochondria; consequently, leading to an improved oxidative capacity (Hood, 2001). Mitochondria biogenesis is stimulated by a number of cellular signalling molecules (e.g., AMPK, CaMK, mitogen-activated protein kinases p38 and p53) which converge to the peroxisome proliferator-activated receptor gamma coactivator 1-aplha (Drake *et al.,* 2016). To date, a number of studies have shown a reduction in the expression of key mitochondrial biogenesis transcription factors as a result of antioxidant administration (Vitamin C – Gomez-Cabrera *et al.,* 2008; Vitamin C and E – Ristow *et al.,* 2009; Vitamin E and ALA – Strobel *et al.,* 2011). This is supported by human research demonstrating a blunting in mRNA and protein content of mitochondria biogenesis factors (i.e. COX4, PGC1-a, NRF1/2) (Paulsen et al., 2014; Morrison et al., 2015). Collectively, there is considerable evidence that AMPK, NRF2 and p38 serve as the intermediate molecules connecting RONS with PGC-1α activation and/or mitochondrial biogenesis markers (Margartielis *et al.,* 2020).

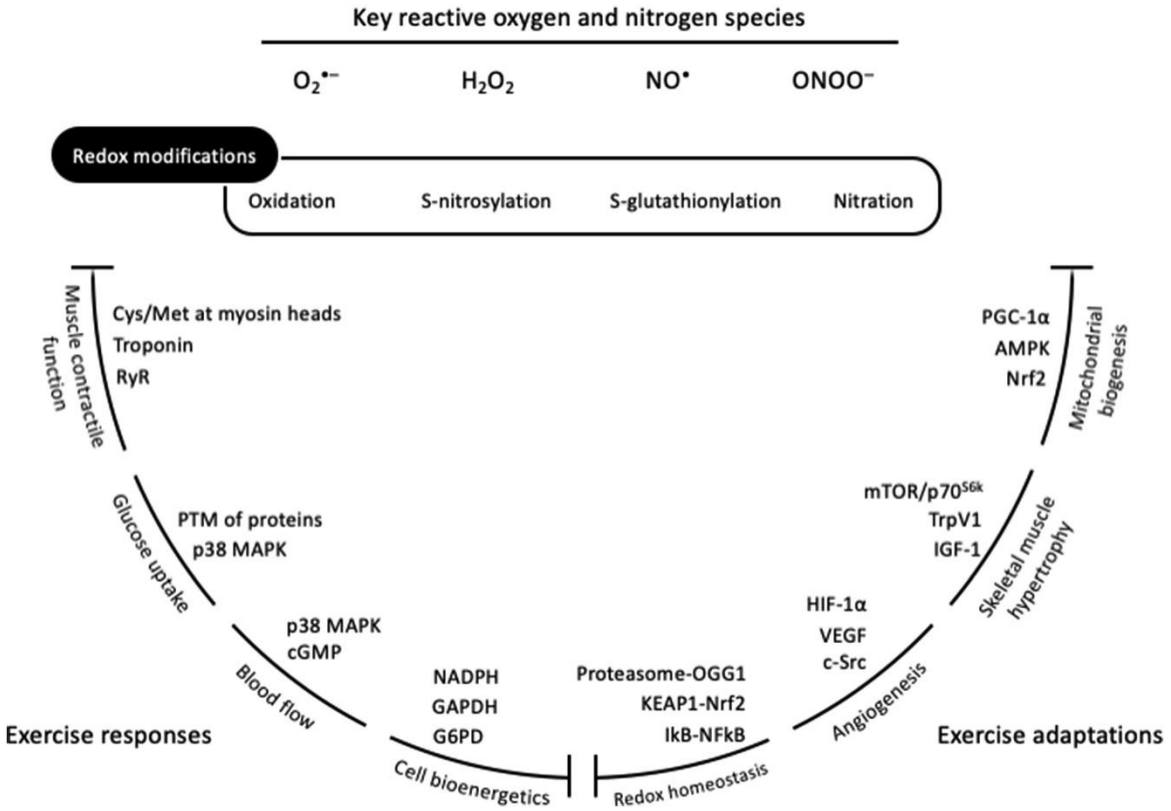
Given the acute responses of reactive species within the regulation of the vascular system as a function of exercise, it is unsurprising that the process of angiogenesis has been examined within redox biology. Exercise is a physiological

stimulus that initiates angiogenesis through a network of mechanisms including mechanical forces (e.g., vascular wall tension, shear stress and strain), tissue hypoxia and cycling hypoxia-reoxygenation phenomena, as well as humoral biochemical factors and accumulation of metabolic by-products (Bloor, 2005); these processes are largely governed by vascular endothelial growth factor (VEGF) and its receptor tyrosine kinases (VEGFR1/2). Although this VEGF/VEGFR complex may not be directly involved within redox signalling, the downstream, kinases, phosphatases, cytokines, and transcription factors are redox-sensitive (Roy *et al.,* 2008). Two of the primary endogenous RONS sources within endothelial cells are the mitochondria and NADPH oxidases, with some particular isoforms (NOX 1,2 and 4) being strongly associated with the angiogenic process. In particular, superoxide, hydrogen peroxide, and NO· produced endogenously or added exogenously in low amounts in cell cultures, have been found to directly up-regulate VEGF expression in diverse cell types (Feliers *et al.,* 2006; *Sen et al.,* 2002; Kim *et al.,* 2006). RONS with signalling properties and adequate diffusion distances, such as hydrogen peroxide, produced during exercise even from extracellular sources (e.g., blood cells) could theoretically induce VEGF expression in endothelial cells and trigger angiogenesis. Taking into account that exercise increases VEGF content, it seems reasonable to speculate that RONS originated from the VEGF-stimulated NADPH oxidases are involved in exercise-induced angiogenesis.

Although much of the research surrounding the redox regulation of exercise adaptation has focused on endurance training, there is accumulating evidence that RONS may also play a role in muscle hypertrophy. It should be noted however that this hypothesis is relatively novel, and the available literature is limited and controversial (Makanae *et al*., 2013; Paulsen *et al.,* 2014; Dutra *et al.,* 2018).

Collectively, the presented research establishes redox reactions as a fundamental part of human biology, and that exercise responses (acute or chronic) are at least partially controlled by redox reactions. An overview of the redox reactions associated with exercise responses and adaptations can be summarised in Figure 2.23.

Figure 2.23. A summary illustrating the central RONS, types of redox modifications and molecules involved in the acute exercise-induced responses and the training-induced adaptations as discussed in the aforementioned sections.

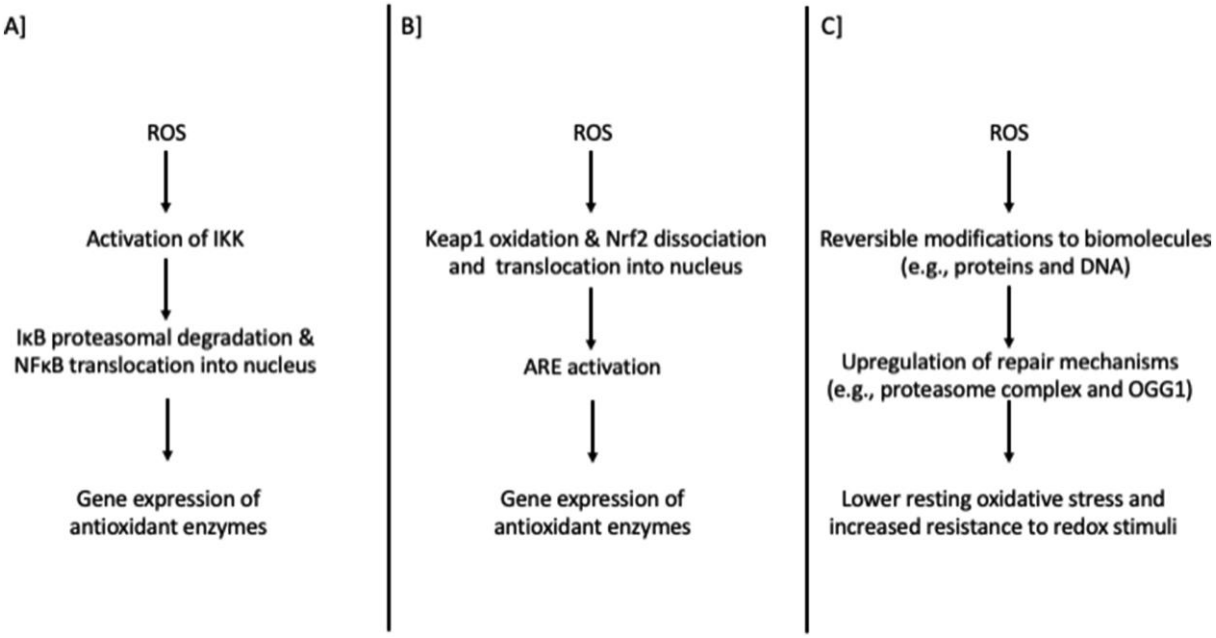


Abbreviations: AMPK, 5' AMP-activated protein kinase; c-Src, proto-oncogene tyrosine-protein kinase; cGMP, cyclic guanosine monophosphate; G6PD, glucose-6-phosphate dehydrogenase; GAPDH, reduced glyceraldehyde 3-phosphate dehydrogenase; H_2O_2, hydrogen peroxide; HIF-1α, hypoxia-inducible factor 1-alpha; IGF-I, insulin-like growth factor 1; IKK, IκB kinase; Keap1, Kelch-like ECH-associated protein 1; LKB1, liver kinase B1; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO•, nitric oxide; Nrf2, nuclear factor erythroid 2-related factor 2; O•-, superoxide radical; OGG1, 8-oxoguanine glycosylase; $ONOO^-$, peroxynitrite; $p70^{S6K}$, ribosomal protein S6 kinase beta-1; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PTM, post-translational modifications; RYR, ryanodine receptors; Trpv1, transient receptor potential cation channel subfamily V member 1; VEGF, vascular endothelial growth factor.

With that being said, one of the most distinctive exercise adaptations regulated by RONS is an improvement in redox homeostasis as indicated by: i) increased gene expression, protein content and activity of antioxidant enzymes, ii) higher resting levels of low molecular weight antioxidants, iii) lower resting levels of oxidative stress biomarkers, iv) increased resistance to oxidative insults, and v) improved repair

mechanisms for oxidatively modified proteins and DNA. Analysing these individual factors goes much deeper than the scope of this thesis and readers are directed towards Margartielis and colleagues (2020) for a comprehensive review; with that being said, an overview of the proposed hypothesis can be observed in Figure 2.24.

Figure 2.4 Proposed mechanisms on how ROS regulate redox homeostasis.



Abbreviations: ARE, antioxidant response element; DNA, deoxyribonucleic acid; IKK, IκB kinase; Keap1, Kelch-like ECH-associated protein 1; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2, nuclear factor erythroid 2-related factor 2; OGG1, 8-oxoguanine glycosylase; ROS, reactive oxygen species.

2.6 Exercise-Induced Oxidative Stress: Experimental Evidence

Based on the definition of oxidative stress, biomarkers associated with the quantification of exercise-induced oxidative stress can be categorised into one of four divisions (Powers & Jackson, 2008): (i) measurement of more stable radicals/oxidants, (ii) small-molecular-weight and/or lipid-soluble antioxidants, (iii) biomarkers associated with redox balance (e.g. S-glutathionylation), and finally, (iv) damaged biological structures associated with oxidative damage (e.g. protein carbonyls, lipid hydroperoxides, DNA damage). Free radicals are extremely elusive, owing to their highly reactive nature; this make them very difficult to examine; thus, measurement techniques typically focus on indirect ‘footprints’ or macrophysiological changes in

response to oxidative damage (Sachdev & Davies, 2008). This section aims to review the evidence associated with exercise and oxidative stress.

2.6.1 Evidence of Exercise and Direct Free Radical Generation

Electron paramagnetic resonance (EPR) spectroscopy is the only technique which allows for the direct quantification of free radical species. EPR is generally used in tandem with spin traps, or probes, which bind to specific radicals, thereby transforming them into their respective adducts; this results in the accumulation of radical-specific signatures which can be detected through EPR spectroscopy (Armstron & Whiteman, 2007). Although numerous spin traps exist, the most popular in the exercise literature is PBN (α-phenyl-*N*-*t*-butylnitrone), and to a lesser extent, its derivative, POBN [α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone].

Davies and colleagues (1982), were the first to use EPR (without a spin trap) where they detected an increase in free radical generation (approximately g = 2.004) from frozen muscle tissue following an exhaustive bout of exercise in rats. This was later confirmed by Jackson *et al.* (1985), who obtained an approximate 70% increase in EPR signal intensity within mouse gastrocnemius muscle following 30 minutes of contractile activity. Ashton *et al.* (1998), was the first to use EPR spectroscopy to report free radical generation following maximal exercise in human serum samples. They observed a three-fold increase in EPR signal of the PBN adduct immediately post-exercise; this was accompanied by an increase in lipid peroxidation via MDA and lipid hydroperoxides. These responses were corroborated by Ashton *et al.* (1999), following incremental exercise to exhaustion; however, supplementation of 1,000mg of ascorbic acid prevented exercise-induced changes in PBN adduct formation and lipid peroxidation. These data were later followed by Davison *et al.* (2002) by using a progressive and incremental exercise to elicit $\dot{V}O_{2max}$. They reported the likely detection of carbon or oxygen-centred lipid-derived alkoxyl radical following exercise; an increase in serum lipid peroxidation was also observed. This supposition has been confirmed by others following incremental exercise to exhaustion, with a correlating increase in free radical outflow (Bailey *et al.*, 2004). It appears this linear relationship between exercise duration/intensity and RONS production is corroborated by other investigators (Ashton *et al.*, 1998; Fogarty *et al.*, 2011; Davison *et al.*, 2012; Parker *et al.*, 2014; Vezzoli *et al.*, 2016). With that being said, following 2-hours of exercise at 55% of $\dot{V}O_{2max}$ in hypoxia, Davison and colleagues (2006) found no selective increase

in secondary free radical concentration compared to normoxic conditions; it should be noted however, that pooled group data indicated the generation of secondary lipid-derived alkoxyl radicals.

The use of EPR has also been applied to human skeletal muscle tissue. Bailey and colleagues (2007) were one of the first to investigate the existence of free radical formation following single leg knee extensor exercise. They confirmed an increase in mitochondrial ubisemiquinone radical formation following extensor exercise performed for 2 min at 50% of maximal work rate and 3 min at 100% of maximal work rate; this was accompanied by detection of lipid-derived radicals and the accumulation of lipid hydroperoxides. This notion was later confirmed in an elderly cohort following maximal knee extensor exercise (Bailey *et al.*, 2010). However, others have investigated the response of exercise training and found no difference in skeletal muscle RONS production via EPR (Shima *et al.*, 2008; Radak *et al.*, 2009).

EPR methodologies are highly versatile and relatively sensitive for the direct measurement of free radicals; on the other hand, EPR isn't without its limitations. For one, there is a lack of sensitivity and a limited number of free radicals detectable *in vivo*; also no DNA-dervied radicals can be detected except with radiation or chemical generation (Mason, 2016). On a more practical level, EPR spectrometers are expensive, and the detection and analysis of the acquired spectra requires solid underpinning theoretical and practical knowledge by the user (Abdel-Rahman *et al.*, 2016). Regardless of these limitations, EPR is an indispensable technique to study exercise-induced oxidative stress.

2.6.2 Evidence of DNA Damage

The link between DNA and exercise is of current interest due to the established relationship with DNA damage and a range of pathological diseases (Jackson & Bartek, 2009). Under periods of metabolic stress, more specifically, during exercise; excessive accumulation of superoxide can liberate the iron from [4Fe-4S] clusters, thus promoting the generation of the hydroxyl radical through Fenton chemistry (Valko *et al.*, 2007). It is thought acute exercise alters transition metal handling (Close *et al.*, 2005) in turn causing hydroxyl-mediated vicinal damage to DNA (Colbey *et al.*, 2015); whereby the promutagenic base, 8-OHdG in DNA is formed from hydroxyl radical attack on 2'- deoxyguanosine (dG), resulting in a hydroxyl moiety replacing the hydrogen atom. When free radicals damage DNA, they attack indiscriminately with the

potential of generating multiple deleterious products; as a result, this suggests there is a high probability of underestimating the total extent of oxidative damage as a function of exercise. Not to mention, the current analytical techniques available can only quantify a fraction of the oxidised modified biomarkers associated with DNA (Nikitaki *et al.*, 2015).

Oxidised bases and/or nucleosides are common biomarkers used to quantify DNA damage as a consequent of exercise; specifically, oxidised guanine in the form of 8-oxoGua and 8-oxodGua are the most commonly measured end products. Okamura *et al.* (1996) revealed that urinary 8-hydroxy-deoxyguanosine significantly increased over an 8-day training camp which required trained athletes to complete 30 ± 3 km/day. These results have been confirmed in sub-elite runners with urinary 8-hydroxy-deoxyguanosine being significantly elevated immediately following 42-km; interestingly, urinary concentrations still had not returned to baseline at the 1-week follow up time point (Tsai *et al.*, 2001). It is also worth noting that prolonged, strenuous ultra-endurance exercise has confirmed the increase in urinary 8-hydroxy-deoxyguanosine following a 6-day period of 11 hours of exercise per day (Poulsen *et al*, 1996), a 2-day ultra-marathon (Miyata *et al.*, 2008), and 4-day supra marathon (Radak *et al.*, 2000). More recently, Davison and colleagues were the first to report a significant increase in mitochondrial 8-OHdG concentration in human skeletal muscle samples following 100 isolated and continuous maximal concentric contractions (Davison *et al.*, 2014). Although these data would suggest exercise of varying intensities, and modalities, can cause an increase in DNA damage, which is then transported and subsequently excreted in the urine, there are a number of discrepancies between methodologies, which can contribute to indirect, and/or unreliable (artefactual) measures; thereby, producing confounded results of 8-OHdG (Poulsen *et al.*, 1999; Knasmuller *et al.*, 2008). This could include artefactual DNA oxidation from ambient oxygen and transition metal ions during the extraction and/or analysis of samples (Finaud *et al.*, 2006; Nie *et al.*, 2013).

In response to these limitations, investigators have used other biomarkers (such as base lesions/AP sites, single- and double-strand breaks, and PCR-based assays) to investigate oxidative damage to DNA (Gonzalez-Hunt *et al.*, 2018). The single cell gel electrophoresis (or ‘comet’) assay is a commonly applied technique to monitor DNA strand breaks associated with exercise (Collins *et al.*, 2008). This is a sensitive and simple technique which is based on the premise that damaged and fragmented DNA

will migrate out of the nucleus (Hartmann *et al.*, 1998). Although this assay is relatively simple to execute, it is incredibly versatile in its application depending on the initial preparation conditions; allowing for the quantification of DNA single/double strand breads, apurinic sites, and purine/pyrimidine oxidation through the use of the lesion specific enzymes endonuclease III (ENDO III) and formamidopyrimidine glycosylase (FPG), which allows for the detection of oxidized pyrimidines and purines, respectively (Collins *et al.*, 1993; Collins *et al.*, 2008).

In a milestone study, Hartmann and colleagues (1994) used the comet assay to detect DNA migration in lymphocytes following an incremental treadmill test to volitional fatigue. They showed that DNA migration peaked at 24-hours following the oxidative insult; interestingly, this damage was still present for up to 72-hours before returning to baseline levels. These results are supported by other similar work (Niess *et al.*, 1996; Mars *et al.*, 1998; Davison *et al.*, 2005; Fogarty *et al.*, 2013; Williamson *et al.*, 2018). These results are not just confined to exhaustive running, as there is evidence which suggests that maximal cycling (Zhang *et al.*, 2004), marathon running (Tsai *et al.*, 2001), Ironman triathlons (Reichhold *et al.*, 2008; Wagner *et al.*, 2010), and rowing (Sardas *et al.*, 2008), all provide a sufficient oxidative challenge to damage DNA. It should be noted, within some of these studies the comet assay detected persistent changes in DNA damage for up to 7-days following initial insult, before returning to baseline values (Hartmann *et al.*, 1998; Tsai *et al.*, 2001). In a landmark study by Peter Moller and colleagues (2001), they observed DNA strand breaks from three days of altitude exposure; this was further compounded by a maximal cycle test to exhaustion in hypoxia. Additionally, it was also observed that ENDOIII-sensitive sites were elevated while no changes were identified in FPG-sensitive sites.

In contrast to the presented research, not all the literature reports oxidative DNA damage in response to exercise. Briviba and colleagues (2005), investigated the oxidative stress response to a half- and full-marathon in 10 healthy amateur runners. They assessed DNA strand breaks, and oxidised purines/pyrimidines using FPG and ENDOIII, respectively. No change was observed in either of the exercise durations across DNA strand breakage or FPG sensitive sites; however, significant increases in the detection of ENDOIII was observed. Additionally, Peters *et al.* (2006) had participants exercising for 2.5-hours at 75% of $\dot{V}O_{2max}$ and observed no change in DNA damage as measured by the comet assay.

Based on the presented research, it appears that exercise of varying modalities and durations causes significant oxidative damage to DNA, with some of the literature suggesting DNA damage peaks at approximately 24-hours (and beyond) following initial insult (Tsai *et al.*, 2001; Mastaloudis *et al.*, 2004; Riechhold *et al.*, 2008). Secondly, select data suggests that this DNA damage is persistent (for up to 7-days following oxidative injury), but is efficiently repaired with no lasting structural changes. It is also important to consider that although some of the results are conflicting, the experimental design and methodologies employed across the studies with regards to exercise duration, modality, intensity, sample size, training status of the subject, and blood sampling timepoints, are inconsistent. Nevertheless, it appears that high-intensity exercise and prolonged endurance exercise cause DNA instability (Davison, 2016; Tryfidou *et al.,* 2019).

Although work examining DNA DSBs is scarce in exercise, their physiological consequences are deleterious with one DSB having the potential to induce genomic instability (Mehta & Haber, 2014). To date, only two studies have investigated whether DNA DSBs appear following exercise. The first study to investigate this was performed by Lippi *et al.* (2016), whereby they assessed four consecutive trials of increasing running distance (5-, 10-, 21- and 42-km) over the course of a year with the primary aim of quantifying DNA double-strand damage in lymphocytes as assessed by detection of *γ*-H2AX foci. They concluded that as exercise distance increased, DNA double stranded damage increased in proportional manner. Although no specific cause of these DSBs was discussed, the same research group followed up by assessing euglycemic and diabetic amateur runners following a half marathon as measured by detection of *γ*-H2AX foci (Lippi *et al.*, 2018). They revealed that at rest there was no difference between groups; however, they reported both healthy and diabetic amateur runners had similar amounts of phosphorylated H2AX in response to 21km of running. It should be noted, the frequency of DSBs within the aforementioned studies is relatively low, especially in the context of genomic- and biodosimetry-based research. An explanation for the lack of frequency of DSBs in the context of exercise, is that endogenous sources of RONS typically produce independent lesions; thus, the probability of these endogenous RONS generating clustered lesions, and subsequent conversion to a DSB, is considerably low (Cannan & Pederson, 2017).

2.6.3 Evidence of Lipid Peroxidation

The first study demonstrating oxidative stress following exercise in humans, was conducted by Dillard and colleagues in 1978. Using expired pentane gas in exercising participants, they observed an approximate 1.8-fold increase during exercise at 75% $\dot{V}O_{2max}$ compared to baseline. Additionally, they also identified that 60 minutes of exercise at 60% $\dot{V}O_{2max}$ induced lipid peroxidation and postulated that vitamin E consumed at a rate of 1,200 IU *dl*-α-tocopherol per day for 2 weeks, decreased basal and exercise expired pentane levels. Accurate measurement of expired pentane is challenging which potential explains why it is rarely used. With that being said, expired pentane (and inferred lipid peroxidation) has been confirmed across the literature (Pincemail *et al.*, 1990; Leaf *et al.*, 1997; Leaf *et al.*, 1999); additionally, Han and colleagues (2000) also revealed a dose-dependent increase in expired pentane in parallel with increasing exercise intensity. In a follow up study by Brady and colleagues (1979), they demonstrated that swimming increased lipid peroxidation in liver and skeletal muscle which was subsequently reduced in the liver as a function of dietary vitamin E supplementation. Although these initial studies provided evidence for exercise-induced lipid peroxidation, the source of free radical production was largely unknown. To date, research has confirmed a multitude of exercise durations, intensities, and modalities, causes a peroxidative response within biological systems; these will be reviewed in the following section.

Davies *et al.* (1982), observed an increase in MDA as a result of exhaustive exercise; this supposition was confirmed in work of Hartmann *et al.* (1995), that revealed incremental, exhaustive treadmill running generated an increase in MDA. Using an incremental, intermittent, sub-maximal cycle ergometer test, Koska *et al.* (2000), observed MDA concentrations increased with a parallel increase in enzymatic antioxidant activity. These results also appear to be true on either extremity of the exercise-intensity continuum with an increase in MDA following not only sprint anaerobic exercise (Marzatico *et al.*, 1997), but also following a half marathon (Child *et al.*, 2000) and an ultra-marathon (Kanter *et al.*, 1988). Similar observations were reported by Dousset *et al.* (2002) and Steinberg *et al.*, (2002) following isometric exercise, whereby circulating TBARS increased following exercise. The notion that exercise increases lipid peroxidation as measured by TBARS has been confirmed by others (Nikolaidis *et al.*, 2007; Nikolaidis *et al.*, 2007; Michailidis *et al.*, 2007). On the contrary, it is worth noting that not all research demonstrates an exercise-induced

increase in MDA; this notion is supported across a multitude of modalities including graded exercise cycling tests (Gaeini *et al.*, 2006; Rahnama *et al.*, 2007; Puspaningtyas *et al.*, 2018), steady state exercise (Morillas-Ruiz *et al.*, 2005), exhaustive running (Niess *et al.*, 1996; Bouzid *et al.*, 2014), rowing (Dernbach *et al.*, 1993) and isometric contractions (Alessio *et al.*, 2000).

As recommended by Cobley and colleagues (2017), the use of TBARS to quantify exercise-induced lipid peroxidation should be abandoned due to the lack of specificity as carbohydrates, sialic acid and prostaglandins can cause artefactual variation within the results (Oh-ishi *et al.*, 2000; Halliwell & Whiteman, 2004). This is more apparent when measuring MDA or isoprostanes specifically as a biomarker of exercise-induced oxidative stress; whereby null findings are more evident across maximal (Davison *et al.*, 2002; Quindry *et al.*, 2003; Bloomer *et al.*, 2007), or submaximal (Orhan *et al.*, 2004; Bloomer *et al.*, 2005; Bloomer *et al.*, 2006) exercise. As a result of this revelation, other measures of lipid peroxidation have been used to quantify exercise oxidative stress. One of the primary products of the propagation step is lipid hydroperoxides which are typically quantified through high performance lipid chromatography, spectrometrically, or enzymatic-based methods via serum and/or plasma. Research by Bailey *et al.* (2001), and Wilber *et al.* (2004), observed an increase in lipid oxidation as indicated by elevated LOOH concentration following cycling protocols comprising of maximal, exhaustive and interval training respectively in hypoxic conditions. Vincent *et al.*, (2004) reported an increase in lipid hydroperoxides following both resistance (7.1%) and aerobic exercise (26.9%). Similar responses in LOOH have also been reported following an acute bout of maximal exercise (Davison *et al.*, 2002; Williamson *et al.*, 2018). Fogarty and colleagues (2013) reported a significant increase in lipid hydroperoxides following 100 isolated and continuous maximal muscle contractions; additionally, this response was attenuated by 14 days of 2x500mg of alpha-lipoic acid supplementation.

On the contrary, there is evidence to suggest no change in circulating lipid hydroperoxides following steady state exercise at 60% HRmax (McClean *et al.*, 2007; McClean *et al.*, 2010). Interestingly, McClean *et al.*, (2015) reported an increase in lipid hydroperoxides following 20 minutes of exercise at 75% of $\dot{V}O_{2max}$, but not at 55% of $\dot{V}O_{2max}$ for 30 minutes, or 100% of $\dot{V}O_{2max}$ for 5 minutes. This may indicate an intensity and/or duration threshold as a function of exercise; similar data has been reported elsewhere (Fogarty *et al.*, 2011; Johnson *et al.*, 2012). More recently, Brown and

colleagues (2018), reported no change in lipid peroxidation as assessed by spectrophotometric analysis of LOOH following 3 x 5 minute bouts of walking at 80% of $\dot{V}O_{2max}$ or 60% of $\dot{V}O_{2max}$ for 30 minutes. In addition, Brown *et al.* (2019), also highlighted lipid peroxidation increased post-prandially following a high fat meal regardless of exercising at 65% of HRmax for 1 hour.

2.6.4 Evidence of Protein Oxidation

In addition to oxidative damage to DNA and lipids, it is evident oxidative attack to proteins or amino acid structures can also occur following exercise. Reznick and colleagues (1992), were one of the first to report an increase in protein oxidation following a single bout of maximal exercise in mice, where it was demonstrated exercise damages proteins and this can be attenuated by consuming a high α-tocopherol diet (10,000IU/kg/day) for 4 weeks.

Radak and colleagues (2003) examined the effects of a super-marathon (328 km) on serum and urinary nitrotyrosine and protein carbonyls across 4 consecutive days. They observed a rapid elevation in protein nitration and carbonylation after the first day of running (93km); this response remained consistent for the subsequent days of competing. Vezzoli and colleagues (2016) confirmed this oxidative damage to proteins following two independent ultra-marathons (50- and 100-km). They showed a linear relationship between exercise duration and oxidative damage; with protein carbonyl accumulation 54% higher in the 50-km group, and 115% higher in the 100-km group compared to rest. These findings have been confirmed in a plethora of other endurance-based studies (Alessio *et al.*, 2000; Goldfarb *et al.*, 2005; Goldfarb *et al.*, 2007; Gochman *et al.*, 2007; Turner *et al.*, 2011; Turner *et al.*, 2014).

Oxidative damage to proteins has also been shown to occur in anaerobic based activities. Bloomer *et al* (2008), observed an increase in protein carbonyls following a Wingate test and subsequent squat test (70% 1RM for 15 repetitions). Interestingly, there was no change in lipid peroxidation or DNA damage following exercise. Moreover, Bloomer and colleagues (2005), reported higher protein oxidation following anaerobic exercise (squatting at 70% of 1RM for 30 minutes) when compared to aerobic exercise (70% of $\dot{V}O_{2max}$ for 30 minutes). It should be noted that these finding conflict with previous research by Goldfarb *et al.* (2002), who observed an increase in protein carbonyls as a function of aerobic exercise. However, it is plausible that 70%

of $\dot{V}O_{2max}$ (as employed by Bloomer *et al.*, 2005) did not cross an intensity and/or duration threshold to induce oxidative damage to proteins.

The body of research does appear to indicate that exercise intensity and/or duration are indeed determinants of protein oxidation. One such study by Lamprecht and colleagues (2008), examined the effects of three independent exercise bouts of 70%, 75%, and 80% of $\dot{V}O_{2max}$; each cycling bout lasting a total of 40 minutes. They concluded post-exercise protein carbonyls increased following exercise at 80% $\dot{V}O_{2max}$; indicating exercise intensity may play a central role in protein oxidation. With respect to duration, Bloomer *et al.* (2007), maintained an exercise intensity of 70% $\dot{V}O_{2max}$ but varied duration between 30-, 60-, and 120-minutes in male and female participants. Plasma protein carbonyl concentration increased across all groups with 120-minutes of cycling showing the highest increase following exercise; protein carbonyl concentration remained elevated for 60-minute post-exercise. Although it appears exercise intensity and duration play a considerable role in the formation of protein carbonyls, others have highlighted the contribution of additional physiological variables (Wadley *et al.*, 2016). In two independent studies, Bloomer and colleagues (2007; 2005), employed an identical exercise protocol (30 minutes at 70% $\dot{V}O_{2max}$) in male cyclists; with the only difference being the training status of the participants [$\dot{V}O_{2max}$: 57 ± 5 ml/kg/min (2007) vs. 45 ± 8 ml/kg/min (2005)]. Although reported as untrained (2005), participants engaged in approximately 3.8 ± 1.8 hours of resistance training per week. However, no difference was observed in the trained group (2007) following 30 minutes of exercise at 70% $\dot{V}O_{2max}$; this is consistent with the literature, indicating trained individuals have a greater potential to mitigate the effects of exercise-induced RONS (Miyazaki *et al.*, 2001; Abed *et al.*, 2004; Gomez-Cabrera *et al.*, 2008; Ji, 2008). Therefore, as reported by others (Radovanovic *et al.*, 2009; Fisher *et al.*, 2011), the novel exercise stimulus, may explain the increase in protein carbonyl concentration following exercise.

Due to the aforementioned reasons, it is unsurprisingly that multiple studies have reported no change in protein carbonyls following exercise (Gaeini *et al.*, 2006; Magalhaes *et al.*, 2007; Shi *et al.*, 2007; Rahnama *et al.*, 2007; Kabasakalis *et al.*, 2011; Fogarty *et al.,* 2013). Based on the above, it is plausible to state that studies examining exercise-induced protein damage should implement protocols of an appropriate duration (>60 minutes) and/or intensity (>70% $\dot{V}O_{2max}$).

2.6.5 Determinants of Exercise-Induced Oxidative Stress

It appears one of the primary determinants of exercise-induced oxidative stress is intensity. Quindry and colleagues (2003) performed four exercise trials of different intensity [(i) maximal exercise to exhaustion; (ii) 45 mins at 10% above lactate threshold; (iii) 45 mins at 10% below lactate threshold, and (iv) 10% below lactate threshold until caloric expenditure equalled trial (ii)] to examine changes in neutrophil activation and oxidative stress. They concluded oxidative stress substantially increased when exercise intensity exceeded the lactate threshold. Similarly, Fogarty *et al.* (2011) demonstrated no change in lipid peroxidation or DNA damage up to 40% $\dot{V}O_{2max}$; whereas measures of oxidative stress significantly increased when exercise intensity exceeded 70% $\dot{V}O_{2max}$. It is important to note that other investigators have chosen to remain consistent with exercise intensity, and instead, make exercise duration the primary variable. Firstly, a study examining the effects of swimming duration and on biomarkers of lipid peroxidation was performed in 1992 by Koe *et al.* concluded that 120 minutes of exercise activated the greatest production of lipid peroxidation in contrast to 60- and 90-minutes. Secondly, Bloomer and colleagues (2007), exercised participants at 70% $\dot{V}O_{2max}$ on a cycle ergometer across a range of exercise durations for a maximum of 120 mins. They reported that protein carbonyl concentration incrementally increased in proportion to exercise duration of fixed intensity. In brief, it remains unknown whether exercise intensity and/or duration is the predominant factor of protein oxidation; nonetheless, it could be postulated that a specific threshold must be exceeded for exercise to cause significant generation of RONS, capable of damaging biologically important structures (Kawamura & Muraoka, 2018).

The final variables associated with exercise-induced oxidative stress relate to training status and antioxidant status of the participants. As discussed in section (antioxidants and exercise-induced oxidative stress), it is clear that regular exercise training can increase endogenous antioxidant capacity, with trained athletes exhibiting greater concentrations of enzymatic- and non-enzymatic antioxidants (Kawamura & Muraoka, 2018). In turn, exhibiting a dampened response to exercise-induced oxidative damage. In addition to endogenous antioxidant status, supplementation of exogenous antioxidants (or lack thereof), may be an important determinant that influences the responses associated with exercise-induced oxidative stress.

2.6.6 Considerations for Hypoxia and Exercise Research

Hypoxia can be characterised as an imbalance between the supply and demand for oxygen (Semenza, 2001); this can occur as a result of environmental manipulation (i.e. terrestrial and simulated altitude), pathological conditions (i.e. ischemia, asphyxia events), or during exercise (Michiels, 2004). Consequently, hypoxia favours the generation of RONS (McGarry, 2018); this is evident during acute (Magalhaes *et al.*, 2004; Pialoux *et al.*, 2009) and chronic (Askew, 2002; Dosek *et al.*, 2007) exposures.

Acute, intermittent, and chronic hypoxia have all demonstrated a relationship with oxidative damage to DNA. Pfeiffer *et al.* (1999), was one of the first to investigate DNA damage as a result of hypoxia; they observed a 2- and 2.1-fold increase in urinary 8-oxodG concentration following 7- and 14-days respectively of winter field training in military personnel. This was accompanied with data from Chao *et al.*, (1999) who observed a 1.3-fold increase in 8-oxodG; both of these studies were performed at altitudes >2500m. Later, Moller and colleagues (2001) conducted a robust study examining the DNA damage response to acute hypoxic exposure (~4500m; ~FiO_2 12%); followed by maximal exercise in hypoxia. They confirmed an increase in single-strand breaks (2.6-fold) following 19-hours of hypoxic exposure, with ENDO III and FPG sites remaining unaltered. With that being said, certain biomarkers of oxidative DNA damaged increased 24-hours following exhaustive hypoxic exercise (single-strand breaks, 2.8-fold; ENDO III, 1.9-fold) while FPG and urinary 8-oxodG remained unaltered. Although the literature varies with regards to exposure times, exercise modalities, intensities, and sample populations, the findings remain largely consistent; exposure to, and exercise within a hypoxic environment induces structural damage to DNA (Schmidt *et al.*, 2002; Lundby *et al.*, 2003; Lundby *et al.*, 2005; Risom *et al.*, 2007).

In addition to DNA damage, the literature also indicates hypoxia-induced effects on biomarkers associated with lipid peroxidation. Simon-Schass & Pabst (1988), produced the first report on exercise-induced oxidative stress at altitude, concluding that expired pentane gas, increases following the initial 4 weeks of a mountain expedition (1.5-fold), which was attenuated by 10 weeks of vitamin E supplementation (2 x 200 mg dl-alpha-tocopheryl acetate). Similar observations have been documented recently by Irarrazaval and colleagues (2017), showing an increase in lipid peroxidation, and a parallel reduction in glutathione peroxidase activity following a 36-hour summit expedition (4220m). These conclusions are supported by other field-

based reports (Vasankari *et al.* 1997; Miller *et al.* 2013; Krzeszowiak *et al.* 2014); consistent across both acute (Magalhaes *et al.*, 2004; Pialoux *et al.*, 2009; Faiss *et al.*, 2013), and chronic hypoxic exposures (Joanny *et al.*, 2001; Askew *et al.*, 2002; Dosek *et al.*, 2007). It should be noted that exercise intensity is significantly compounded at altitude due to altered mitochondrial PO_2. For one, Buskirk and colleagues (1967), investigated the effect of slow hiking above 8000m, and concluded an elevation-dependent increase in exercise intensity at approximately 100% of relative $\dot{V}O_{2max}$. Collectively, it appears that environmental hypoxia and exercise contribute to oxidative stress in a dose-dependent manner (Goto *et al.*, 2003; Debevec *et al.*, 2015).

With respect to the mechanistic sources aligned to the production of RONS in hypoxia, the body of literature remains uncertain (Debevec *et al.*, 2017). That said, there does appear to be a number of mechanisms favourably associated with hypoxia. Reductive stress (Duranteau *et al.*, 1998), catecholamine production (Mazzeo *et al.*, 1998), augmented mitochondria redox potential (Kehrer & Lund, 1994), activation of xanthine oxidase (Yuan *et al.*, 2004), an impairment in antioxidant capacity (Quindry *et al.*, 2016), and exposure to ultra-violet light (at terrestrial altitude) have a greater propensity to generate RONS in hypoxia. It is also worth noting that exposure time within a hypoxic environment, potentially explains the mechanistic effect following acute hypoxia versus chronic exposure (Moller *et al.*, 2008). Acute hypoxia appears to induce a state of reductive stress due to the inability to transfer electrons to oxygen (Kehrer & Lund, 1994; Askew, 2002). Additionally, mitochondrial complex III of the electron transport chain is involved in oxygen sensing and produces superoxide to stabilise hypoxia inducible factor-1α (Guzy & Schumacker, 2006). On the other hand, oxidative stress as a result of prolonged hypoxia appears to activate an inflammatory response which can contribute to oxidative damage to DNA and lipids (Ghezzi *et al.*, 1991; Karakurum *et al.*, 1994).

Although hypoxia is known to increase the accumulation of RONS, thereby inducing oxidative damage to DNA and lipids, it appears this response is exacerbated in hypobaric hypoxia in contrast to normobaric conditions (Loeppky *et al.*, 1997; Savourey *et al.*, 2003; Faiss *et al.*, 2013). Faiss and colleagues (2013) observed a significant increase in advanced oxidation protein products and enzymatic antioxidants as a function exercise in hypobaric hypoxia (3000m); this was combined with a reduced plasma $NO\cdot$ bioavailability and a lower pH concentration, as confirmed by others (Guzy & Schumacker, 2006; Thomas *et al.*, 2008; Pialoux *et al.*, 2011). This combined

hypoxic- and exercise-driven acidosis, creates a favourable environment for the peroxynitrite-mediated generation of the hydroxyl radical (Kellum *et al.*, 2004; Hassan *et al.*, 2009). More recently, this hypothesis has been supported in a number of comparative studies between normobaric- and hypobaric-hypoxia. Debevec *et al.* (2015), reported an increase in plasma concentrations of nitrotyrsine, superoxide dismutase, and uric acid following hypobaric hypoxia. In addition, Ribon *et al.*, (2016) reported a similar response with MDA, superoxide dismutase, glutathione peroxidase and catalase all increasing to a greater extent following hypobaric hypoxia. It appears that hypobaric conditions not only cause an increase in oxidative damage, but also modifies the endogenous antioxidant capacity (Saugy *et al.*, 2014). Mechanistically, there are three distinct physiological responses which favour hypobaric-mediated RONS formation in contrast to normobaric. For one, there is a greater alveolar dead space in hypobaric conditions, thus lending to a lower tidal volume, hypocapnia, and concurrent respiratory frequency (Savourey *et al.*, 2003; Faiss *et al.*, 2013). Secondly, Bailey and colleagues (2001), reported a negative correlation between oxygen saturation and oxidative stress in hypobaric hypoxic; potentially inducing greater hypoxemia. Finally, Hemmingsson & Linnarson (2009), suggested a greater retention of NO· in hypobaric conditions, as lower expired NO· was measured.

2.6.7 Antioxidants and Exercise-Induced Oxidative Stress

Given the role of antioxidants in biological systems, it is logical to; (i) examine the antioxidant response to oxidative stress, and (ii) explore the potential ramifications of antioxidant supplementation and exercise. One of the initial approaches to examining exercise-induced oxidative stress was quantifying total antioxidant capacity (TAC). This assay is based on the premise that Trolox, an analog of vitamin E, will react with oxygen subsequently altering lipid peroxidation via the peroxyl radical within the designated biological sample (Arts *et al.*, 2004). It should be noted, this assay is fundamentally flawed and should be abandoned in exercise-related studies (Arts *et al.*, 2004; Pompella *et al.*, 2014; Cobley *et al.*, 2017). The TAC assay lacks specificity due to; (i) artefactual data errors due to supra-physiological exposure to environmental oxygen (Cobley *et al.*, 2017), and (ii) exacerbated results as a function of purine metabolism (Ghio *et al.*, 2005). Nevertheless, several studies have historically employed the used of this assay to examine exercise-induced modifications to oxidative stress (Childs *et al.*, 1999; Liu *et al.*, 1999; Briviba *et al.*, 2005). With that

being said, this section will focus on reviewing the literature directly relating to this thesis.

Production of RONS can lead to several modifications to DNA which have been linked to carcinogenesis, aging, and cardiovascular diseases (Birben *et al.*, 2012); thus, it is unsurprising that several studies have investigated the use of antioxidant supplementation to mitigate exercise-induced DNA damage. Hartmann and colleagues (1995), reported an increase in lymphocyte DNA damage 24-hours following exhaustive exercise; this increase in DNA damage as quantified using the comet assay, was attenuated following 1200mg of vitamin C supplementation for 14-days prior to exercise. Following this, Sumida *et al.* (1997) demonstrated the ability of daily beta-carotene supplementation (30mg) for 30 days to attenuate DNA damage as quantified by urinary 8-OhdG following an incremental exercise test to exhaustion. This was corroborated in the data of Morillas-Ruiz *et al.* (2005), and Bloomer *et al.*, (2006) following steady-state exercise (90 minutes of cycling at 70% $\dot{V}O_{2max}$ and 20minutes of running at 70% $\dot{V}O_{2max}$, respectively) when combinations of antioxidants were consumed. With that being said, other investigators have reported no effect of supplementation, and indeed may actually contribute to antioxidative stress.

Following administration of an antioxidant cocktail, Davison and colleagues (2005), concluded that supplementation did not selectively mitigate DNA damage induced by an incremental running test to exhaustion. Similarly, Mastaloudis *et al.*, (2004) reported no effect of antioxidant supplementation over the course of an ultramarathon race. Some of the pioneering research in this area by Dillard *et al.* (1978) and Sumida *et al.* (1989) observed that supplementing with a single antioxidant (vitamin E for 2- and 4-weeks, respectively) resulted in an increase in biomarkers associated with oxidative stress. These are not the only reports of antioxidant supplementation inducing a pro-oxidant effect (Kontush *et al.*, 1996; Nieman *et al.*, 2004; Lamprecht *et al.*, 2009); these results have been correlated across different exercise modalities including Ironman Triathlon (Nieman *et al.*, 2004), ultra-endurance training (Knez *et al.*, 2007), and an intermittent shuttle run (Bailey *et al.*, 2011). It is thought this may be due to an overconsumption of antioxidants, thereby inducing a disturbance in redox biochemistry (Vina *et al.*, 2010).

As a result, foods with high concentrations of antioxidants have been used to offer cellular protection without promoting pro-oxidant effects (Lyall *et al.*, 2009). For one, Fogarty and colleagues (2013) reported acute and chronic supplementation of

watercress attenuated DNA damage following an incremental exercise treadmill test to exhaustion. In addition, although DNA damage was not measured, Bell *et al.* (2014) observed a reduction in oxidative stress and inflammatory markers following Montmorency cherry juice consumption. Finally, Bloedon and colleagues (2019), recently published a systematic review and meta-analysis indicating fruits rich in anthocyanins (pomegranates, grapes, chokeberry, tart cherry etc.) have a significant, yet small effect, of reducing exercise-induced oxidative stress.

The vast majority of the research has attempted to investigate the ability of antioxidants to curb exercise-induced free radical production; in turn, reducing oxidative damage to biomolecules. However, to date, the necessity to use synthetic- and/or natural-antioxidant products to mitigate exercise-induced oxidative damage remains contentious. One such contention, is that during non-exhaustive, moderate exercise, free radicals activate signalling pathways of the endogenous antioxidant system; whereby increasing the activity of superoxide dismutase and glutathione peroxidase (Ji, 2002; Ji *et al.*, 2006). By using exercise as an antioxidant *per se*, increases in glutathione peroxidase and catalase can regulate hydrogen peroxide concentrations, whereas adaptations to superoxide dismutase can reduce the potential of hydroxyl radical formation through the Haber-Weise pathway (Ji, 2007). This adaptive response is thought to occur through several ways; (i) Activation of kinases such as MAPK, (ii) Hydrogen peroxide-mediated inhibition of protein tyrosine phosphate and simultaneous activation of NFκβ inducing kinase (Li & Engelhardt, 2006), and (iii) RONS potentially regulation of the synthesis and degradation of transcription factors; for example, activating-protein 1 (Hoffmann *et al.*, 2005). It is clear that activation of these pathways via chronic exercise training, induces changes to not only SOD and GPx, but also inducible nitric oxide synthase (Yavari *et al.*, 2015); this has been corroborated by others (Arikawa *et al.*, 2013; Mitranun *et al.*, 2014). Collectively, the research supports the notion that aerobic training improves the efficiency of the endogenous antioxidant system, thereby leading to a greater mitochondrial capacity to scavenge free radicals (Chandwaney *et al.*, 1998) and a reduction in superoxide production by the mitochondrial membrane potential at basal conditions (Daussin *et al.*, 2012; Camiletti-Moiron *et al.*, 2013). With that being said, it has been argued that if a hormetic response is required, and indeed beneficial, to the oxidative-antioxidant homeostasis in the cell, the attenuation and/or scavenging of

exercise-induced free radicals via antioxidant supplementation may dampen this ability (Gomez-Cabrera *et al.*, 2005; Ji, 2006).

Given the presented literature, it would appear the general consensus seems to advocate the consumption of antioxidants through a varied and balanced diet to achieve the optimal antioxidant status of individuals participating in exercise (Peternelj & Coombes, 2011). For example, diets rich in Allium vegetables reduced the GSH:GSSG ratio following exercise training (Choi & Cho, 2006). Similarly, following an antioxidant-restricted diet, participants exercised sub-maximally and to exhaustion; F_2-isoprostanes was 38% and 45% higher respectively in the antioxidant restricted group in comparison to the control (Watson *et al.*, 2002). Peternelj & Coombes (2011) presented consistent data indicating antioxidant supplementation may indeed prevent exercise-induced oxidative stress, but high concentrations of antioxidant supplementation blunt the adaptive response of exercise; this has been reported by others (Khassaf *et al.*, 2003; Fischer *et al.*, 2006). The recommended intake of ascorbic acid is approximately 200mg/day which can be achieved through daily fruit and vegetable intake (Frei *et al.*, 2012); furthermore, it has been reported that male and female athletes have a daily intake upwards of 520mg and 230mg respectively (Food and Nutrition Board, 2002). However, with that being said, studies using over 1000mg (Gomez-Cabrera *et al.*, 2008), or indeed up to 3000mg per day (Braakhuis *et al.*, 2014), can not only reduce oxidative stress, but also lead to impairments in performance, mitochondrial biogenesis, and training adaptations. In consideration of the literature (Peternelj & Coombes, 2011; Pingitore *et al.*, 2015), it would seem there is no shared agreement in relation to exogenous antioxidant supplementation (Draeger *et al.*, 2014).

2.6.8 Physiological Adaptation of Exercise and Antioxidants

It has been proposed that supplementation of an antioxidant substance may improve exercise performance by promoting muscular contraction and power output (Kubukeli *et al.*, 2002; Laursen and Jenkins, 2002). However, more recently it has been suggested that exogenous antioxidant supplementation may interfere and blunt the beneficial adaptations to exercise (Venditti *et al.*, 2014). The general consensus remains disputatious, with some reports indicating exogenous antioxidant supplementation prevents adaptations associated with exercise, such as angiogenesis, mitochondrial biogenesis, and hypertrophy (Watson *et al.*, 2005), while other investigators have reported conflicting findings to this supposition. Reid (2001)

was the first to demonstrate a relationship between RONS production and muscle function; this corroborates the theory of hormesis (Yun & Finkel, 2014). Mechanistically, it is thought administration of antioxidants such as ascorbic acid and/or α-tocopherol, scavenge free radicals by proton donation; thereby, weakening the RONS-mediated signalling for metabolism, mitochondriogenesis, and other adaptations associated with exercise (Ristow, 2014; Lamprecht, 2015).

During muscular contraction and/or mechanical distention, superoxide and NO· are the primary RONS generated by skeletal muscle tissue (McArdle & Jackson, 2000); which have the potential to induce changes in gene expression via activation of cell signalling and regulatory pathways (Droge, 2002; Jackson *et al.*, 2002). Some of the early work published from the laboratory of Malcolm Jackson indicated acute muscle contraction increased the activity of SOD, catalase, and heat shock protein-70 (McArdle *et al.*, 2001); this was also corroborated within human skeletal muscle tissue (Khassaf *et al.*, 2001). It was later hypothesised that this adaptive response to exercise was mediated via RONS after incubation of hydrogen peroxide failed to increase SOD, catalase, and HSP60/70 following 8 weeks of $500mg^{-1}$ of vitamin C supplementation (Khassaf *et al.*, 2003). It has subsequently been reported in rodents that antioxidant supplementation attenuated exercise-induced adaptations in mitochondrial biogenesis (Gomez-Cobrera *et al.*, 2008a; Strobel *et al.*, 2011; Abadi et al, 2013); interestingly, others has reported that these responses did not affect total mitochondrial mass (Abadi *et al.*, 2013; Meier *et al.*, 2013). These findings have also been confirmed in humans with Ristow and colleagues (2009) reporting a decrease in SOD1, SOD2, GPx, and measures of insulin sensitivity following 4 weeks of vitamin E and C supplementation. Administering the same antioxidant combination, Paulsen *et al.*, (2014) demonstrated a reduction in biomarkers associated with mitochondrial biogenesis following an exercise program intervention. With that being said, not all studies examining the role of antioxidant supplementation on exercise adaptation have reported impediments to cell signalling and redox sensitive pathways (Nieman *et al.*, 2010; Yfanti *et al.*, 2010; Petersen *et al.*, 2012).

Nevertheless, the premise of the underpinning theory suggests exogenous antioxidants (namely ascorbic acid and α-tocopherol), scavenges exercise-induced RONS; in turn, preventing the activation of redox signalling pathways associated with exercise adaptation (Cobley *et al.*, 2015). It is clear within the literature that exercise activates signalling proteins (MAPK; Wadley *et al.*, 2013), transcription factors (AP-1,

NRF2; Gomez-Cabrera *et al.*, 2008; Reynaert *et al.*, 2004) and co-activators (PGC-1α; Kang *et al.*, 2009) via the generation and interaction with superoxide, NO·, peroxynitrite, and hydrogen peroxide. It is important to emphasize, that this area is a relatively recent niche within the area of exercise redox biology with arguably an underappreciation for the mechanistic nature of redox signalling associated with exercise adaptation, and exogenous antioxidant supplementation. One of the major limitations associated with this research and attempting to draw robust conclusions, is the compartmentalized nature of RONS signalling, and lack of specificity associated with targeted antioxidant therapies (Merry & Ristow, 2016). With that being said, there are a few reports which have attempted to elucidate this. Ito and colleague (2013) identified NO·, peroxynitrite and Ca^{2+} as activators of intracellular signalling pathways with increased muscle hypertrophy. Additionally, by inhibiting the NADPH-oxidase-mediated generation of superoxide, the muscle hypertrophic responses were blunted. Secondly, Min and colleagues (2011) have published data indicating the mitochondrial-targeted antioxidant, SS-21, prevented immobilisation-induced muscle atrophy. The development of mitochondrial- and more so, organelle-targeted antioxidants will provide a more comprehensive understanding of the location, type, and target sites of exercise-induced RONS, and how best to administer antioxidants to improve exercise performance without blunting the adaptation response (Merry & Ristow, 2016).

2.7 Summary of Literature

The concept of hormesis demonstrates that RONS are generated as a result of normal physiological functions such as cell signalling and exercise adaptation (Powers & Jackson, 2008; Halliwell & Gutteridge, 2007); however, oxidative damage to important biological molecules and downstream disruption to cellular redox biology can occur (Sies & Jones, 2007). This phenomenon known as oxidative stress is strongly implicated in disease etiology and pathology (Booth *et al.*, 2017). It is therefore unsurprising that investigators have focused their attention on the administration of antioxidant supplementation to attenuate the detrimental effects associated with RONS (Fogarty *et al.*, 2011). With that being said, much of the somewhat recent evidence would suggest RONS mediate the beneficial adaptations associated with exercise (Venditti *et al.,* 2014), and the consumption of certain antioxidants may attenuate these effects (Ristow *et al.,* 2009). Furthermore, it does seem apparent that the mechanism of delivery of such antioxidants is implicated in the downstream effects. For instance,

the general recommendation is that athletes eat diets rich in plants and fruits in order to achieve the beneficial effects of antioxidants; this appears to provide a greater provision of essential micronutrients. On the other hand, athletes are discouraged from the consumption of pill-based antioxidants which are typically highly concentrated and as a result, rapidly increases *in vivo* plasma concentrations; this has the potential to cause a prooxidant effect, sometimes referred to as antioxidative stress (Wray *et al.,* 2009; Gomez-Cabrera *et al.,* 2012; Villanueva *et al.,* 2012). With all things considered, targeted supplementation although used over the last decade within clinical populations, has been somewhat absent from the area of exercise-induced oxidative stress (Shill *et al.*, 2016). To the best of the authors knowledge, no study has outlined the effects of mitochondrial-targeted supplementation on biological indices of oxidative damage; specifically, to the mitochondrial genome.

It is clear that exercise of sufficient duration and/or intensity induces structural damage to DNA (Neubauer *et al.*, 2008). If left unrepaired, or indeed if repaired incorrectly, there is the potential to induce mutagenesis and genomic instability (Liu *et al.*, 2018); as such, there is a greater need to understand the DNA damage-repair response. For the most part, exercise-based research examines the acute effects of exercise-mediated oxidative damage to DNA (Baker *et al.*, 2004; Bailey *et al.*, 2007; Yasuda *et al.*, 2015, Soares *et al.*, 2015) with very little evidence investigating the repair capacity following an oxidative insult. Additionally, DNA damage is usually quantified as single-strand damage, with only a minority of studies investigating base oxidation (Briviba *et al.,* 2005; Reichhold *et al.,* 2008; Soares *et al.*, 2015). Due to the highly damaging, undiscriminating nature of the hydroxyl radical, a multitude of DNA lesions can occur; arguably, the most severe lesions are double-strand breaks, with a single double-strand break containing the potential to activate cell death. It is thus surprising, that only a few exercise-based studies have quantified the extent of double-strand DNA damage as a function of exercise (Lippi *et al.*, 2016; Lippi *et al.*, 2018); additionally, neither of these studies have attempted to observe the repair response to these genotoxic lesions.

In addition to the damaging effects of exercise on DNA, terrestrial and/or simulated hypoxia has been demonstrated to compound the oxidative damage response (Lundby *et al.*, 2005; Risom *et al.*, 2007). Moller *et al.* (2001), observed an increase in oxidative DNA damage as a result of acute hypoxia exposure which was partially exacerbated (single strand breaks and ENDO III only) by exhaustive hypoxia

exercise (~4500m; ~FiO_2 12%). With that being said, research examining the DNA damage-repair response in hypoxia is warranted. Furthermore, to date, no research has identified the possible induction of DNA double-strand breaks following hypoxic exercise; or indeed, the repair of double-strand DNA damage as a function of high-intensity hypoxic exercise.

As previously mentioned, much of the research has investigated oxidative stress and general/systemic antioxidant supplementation, either as single entities, or combinations of antioxidants. However, given the physiological contribution of mitochondria to bioenergetics, apoptosis, redox regulation, and antioxidant defences, it is surprising that the role of exercise redox biology for the most part, has received considerably less attention (Chan, 2012; Hoitzing *et al.*, 2015). Furthermore, mitochondrial-derived RONS have direct signalling roles associated with thiol/redox signalling proteins and transcription factors; not to mention the emerging evidence of retrograde signalling from the mitochondrial genome to the nuclear domain (Sena & Chandel, 2012; Quinlan *et al.*, 2013). As such, in the same way antioxidant supplementation has the potential to attenuate the beneficial effects of exercise by scavenging systemic RONS, it would be logical to investigate whether mitochondrial targeted supplementation abrogate the positive, physiological roles associated with mitochondrial RONS production.

2.8 Development of Null Hypothesis

The following section details the primary experimental variables aligned to this work. All hypotheses have been developed based on the current body of evidence as presented in the forgoing chapter. Thus, depending on the results yielded from the statistical analysis of the experimental data, rejection, or acceptance of the hypothesis at the level of significance will be synthesised.

2.8.1 Experimental Hypothesis

Study 1 - *Exogenous Plant-Based Nutraceutical Supplementation and Peripheral Mononuclear Cell DNA Damage Following High Intensity Exercise*

H_0 = Maximal, exhaustive exercise will not induce a state of oxidative stress as confirmed via biomarkers.

H_0 = A plant-based nutraceutical will not attenuate biomarkers associated with exercise-induced oxidative stress.

Study 2 - *The DNA Damage-Repair Response and Systemic Oxidative Stress as a Function of High-Intensity Hypoxic Exercise*

H_0 = High-intensity exercise will not induce DNA damage in hypoxia

H_0 = Exercise-induced DNA damage will not be efficiently repaired following hypoxic exercise

Study 3 – *Acute and Chronic Administration of Mitochondrial Targeted Quinone and Mitochondrial DNA Damage following High-Intensity Intermittent Exercise*

H_0 = High-intensity intermittent exercise will not induce lymphocyte and human muscle mitochondrial DNA damage.

H_0 = Acute and chronic administration of a mitochondrial-targeted antioxidant (MitoQ) will not attenuate mitochondrial DNA damage following exercise.

Chapter Three

Chapter 3:
General Methodology

3.0. Conceptual Considerations

3.0.1 Ethical Approval

The Ulster University Research Ethics Committee (UUREC) granted permission for all data collection. All sample collection, storage and analysis of human tissue was completed in accordance with the Human Tissue Act 2004. (See Appendix A for ethics documentation).

3.0.2 Participant Recruitment

Participants were recruited from the general Ulster University population and local sports clubs. All Ulster University students and staff were recruited via the university email network (Appendix B) and verbal communication. Participant characteristics and corresponding inclusion/exclusion criteria are outlined in appropriate experimental chapters. All participants were recruited as per defined inclusion and exclusion criteria. Common inclusion criteria included healthy, recreationally trained (3-5 aerobic and/or anaerobic sessions per week) males aged 18-35. All participants were non-smokers and free from medication and/or antioxidant supplementation. Volunteers who expressed interest in participating were provided with a detailed information sheet (Appendix C), and given sufficient time to read the information document and ask for clarification. A 2-week ‘cooling off’ period from initial contact was provided to all potential participants prior to gaining consent. All participants provided written informed consent (Appendix D) for the collection of biological material in accordance with the Declaration of Helsinki (2002).

3.1 Laboratory Considerations

3.1.1 Environmental Conditions

For study one and three, room temperature and humidity were controlled and measured using an internal air conditioning unit (Sanyo, China). Barometric pressure was obtained from a wall mounted barometer. Study two required the use of an acclimatisation chamber to expose participants to normobaric hypoxia, and normoxic conditions; participants were blinded to the condition. Participants entered the environmental chamber (Design Environmental Ltd., Wales) in combination with

Contour programming and a logging software package (Contour Software, Design Environmental Ltd., Wales). Participants completed the exercise trials in normoxic fraction of inspired oxygen (F_IO_2) = 0.21% and within a hypoxic environment (F_IO_2) = 0.12%. Temperature and humidity were controlled at 16°C and 50% respectively across all trials associated with the acclimatisation chamber.

3.1.2 Oxygen Saturation

A finger pulse oximeter (Merlin M-Scope, Pulse Oximeter, Medscope, UK) was used to determine arterial oxygen saturation by calculating oxygenated haemoglobin and non-oxygenated haemoglobin. The technique uses wavelengths of 940 nm of infrared, and 660 nm of red light, emitted through diodes. The oxygenated haemoglobin absorbs the infrared light whereas the red light is absorbed by the non-oxygenated haemoglobin.

3.1.3 Packed Cell Volume

Whole blood was placed into a 75 µl herpaninised capillary tube (Hawksley and Sons Limited, Sussex, UK) and sealed at the distal end with cristaseal (Hawksley and Sons Limited, Sussex, UK). The sealed tubes were placed into a micro haematocrit centrifuge with the sealed end facing outwards. Samples were spun at 11,800 RPM for 4 minutes. Upon spin completion, packed erythrocytes were measured using a Hawksley Micro Haematocrit Reader (Hawksley and Sons Limited, Sussex, UK). Two samples for each participant were measured and the mean value calculated. Whole blood values were quantified in L^{-1} and subsequently corrected by 1.5% for plasma trapped in erythrocytes (Dacie and Lewis, 1968).

3.1.4 Haemoglobin

The Sysmex Analyser 9000 (Vector Scientific, Belfast, Northern Ireland) was used to quantify the concentration of haemoglobin in whole blood. The Sodium-Lauryl Sulphate method (SLS-Hb) was used to determine haemoglobin concentration.

3.1.5 Plasma Volume Changes

Dill and Costill (1974) equations were used to calculate plasma volume changes:

$BV_{post} = BV_{pre}\ (Hb_{pre}/Hb_{post})$

$CV_{post} = BV_{post}\ (HCT_{post}/100)$

$PV_{post} = BV_{post} - CV_{post}$

$PVC\ (\%) = 100((PV_{post}\text{-}PV_{pre}) / PV_{pre})$

3.1.6 Anthropometric Measurements

Standard anthropometric measurements were taken during the familiarisation phases of each study. Participants were asked to remove footwear, wear shorts and a t-shirt. Height was measured using a free-standing stadiometer (Seca, Cardiokinetics, Salford, UK) to the nearest 0.1cm. Body mass was measured to the nearest 0.1kg. Calibration of the stadiometer was carried out with a 1kg free weight (mass) and a tape measure (height).

3.2 Cardiovascular Measurements and Methods of Exercise

3.2.1 Laboratory Heart Rate

Heart rate during exercise was determined using an electrocardiograph (ECG) calibrated short range telemetry device (Polar Electro Oy, Finland). This is comprised of an adjustable belt containing two electrodes, which were placed either side of the participants' sternum. This transmitting device corresponds to a specific wrist-watch, which allows continuous wireless reception and internal recording of heart rate expressed in beats per minute ($b.min^{-1}$).

3.2.2 Cardiopulmonary Exercise Testing

A Quark (CPET, Cosmed, Italy) metabolic cart was used to provide breath-by-breath measurements of pulmonary gaseous exchange including oxygen consumption, carbon dioxide exhalation and ventilation rate. Subsequently, this provided data identifying oxygen kinetics and respiratory quotients in response to the exercise tests used in the subsequent chapters. The flowometer turbine was calibrated using a syringe to replicate inspiration and expiration on the morning prior to each experimental visit. The sampling line was calibrated before each participant to the manufactures recommended gas mixture (O_2 16%, CO_2 5%, N_2 Bal).

3.2.3 Assessment of $\dot{V}O_{2max}$

Maximal aerobic exercise tests were employed to determine work rate for experimental exercise protocols. Pilot testing was used to confirm the validity and safety of both maximal aerobic tests and experimental steady state exercise protocols. The measurement of maximal oxygen uptake is historically described by Taylor *et al.* (1955) and is recognised as the gold standard to determine a true $\dot{V}O_{2max}$. With that being said, the classic plateau is difficult to ascertain within a practical setting; as a result, many researchers have applied additional end criteria such as RER (≥ 1.0 (Paterson *et al*., 1999); 1.1 (Brown *et al*., 2002); 1.15 (Issekutz *et al.,* 1962)), specific percentages of age-adjusted heart rate max (American Thoracic Society, 2003; Jackson *et al.,* 2009), high post-exercise lactate concentrations (Astrand *et al.,* 1973), or the participants rate of perceived exertion (Church *et al.,* 2008). Currently, there are no consensus regarding the assessment of maximal effort during a continuous graded exercise test (Edvardsen *et al.,* 2014; Schaun, 2017); thus, a combination of the aforementioned variables has often been used to reduce variability (Edvardsen *et al.,* 2013). Given the presented literature, the experimental studies within this thesis applied the following criteria to define $\dot{V}O_{2max}$: a plateau of x ml·kg·min in $\dot{V}O_{2max}$, an RER of >1.15 (arbitrary units), an RPE > 17 (Borg Scale$_{6\text{-}20}$ rating), and a maximal heart rate within ± 10 beats per minute. The protocols used for specific $\dot{V}O_{2max}$ testing are outlined in each experimental chapter.

3.2.4 Treadmill

For study 1, the same treadmill was used for all trials (Pulsar motorised treadmill, H-P Comos, Hamburg, Germany). Before data collection, each study had a familiarisation phase which allowed participants to become habituated to the exercise. The protocol was specifically designed to be progressive and incremental to elicit $\dot{V}O_{2max}$. Treadmill speed was set at 11 km/h with a 1% rise in elevation at 1-min intervals until volitional fatigue.

3.2.5 Cycle Ergometer

For study 2 and 3, participants cycled at a cadence of 70-90 revolutions per minute on a friction-braked cycle ergometer to produce a power output of equivalent to their bodyweight. The workload was increased by 0.5 Watts per kg of body weight every 2

minutes until the participant could no longer maintain the required work rate (Jamnick *et al.*, 2018).

3.2.6 Rate of Perceived Exertion

The Borg Scale (1973) was used to determine rate of perceived exertion (RPE). The Borg Scale uses a numeric value from 6-20 where participants indicate their RPE from "very very light" to "maximum". Upon completion of all $\dot{V}O_{2max}$ tests, all participants scored ≥19 RPE.

3.3 Haematological Measurement

3.3.1 Venous Blood Collection Procedure

Participants were asked to assume the supine position on an appropriate medical bed. A quick release tourniquet was applied to the distal region of the participants humerus with appropriate restriction. Upon selection of an appropriate antecubital vein, a 70% v/v isopropyl alcohol wipe was used to clean the area. All blood samples were collected using the Vacutainer™ method (Becton, Dickinson, Oxford, UK).

Blood samples were collected using di-potassium ethylene diamine tetra-acetic acid (EDTA) vacutainers, which act as anticoagulants via sequestering of calcium ions (Lundblad, 2005). EDTA tubes were placed in the 4°C fridge to inhibit DNA repair-enzyme activation. For the purpose of analysing and storing lymphocytes for DNA damage analysis, 3ml of whole blood was layered onto 3ml of Histopaque (Sigma) in a 15ml centrifuge tube. Samples were centrifuged for 30 minutes at 3500 RPM at 4°C. 1ml of mononuclear blood cells were aspirated into a separate 15ml centrifuge tube along with 10ml of PBS, and centrifuged for 10 minutes at 3500 RPM to create a pellet; a further PBS wash was conducted. The pellet was re-suspended in 800 µl RPMI, 100 µl FCS, 100 µl DMSO with a 1.5ml Eppendorf tube for storage at -80°C.

Advanced serum separating tubes (SST) were used in order to form a fibrin clot; thus, removing the fibrogen, platelets and blood proteins. Serum was used for the primary analysis of lipid derived metabolites associated with oxidative stress as there is evidence to suggest these have a greater stability (Boyanton & Blick, 2002). SST tubes were allowed to clot at room temperature before being centrifuged for 10 minutes at 3500 RPM. Plasma and serum samples were aliquoted into 1.5ml Eppendorf tubes using a 1ml pipette, and frozen immediately at -80°C for analysis (within six months).

3.4 Biochemical and Molecular Techniques

3.4.1 The Comet Assay

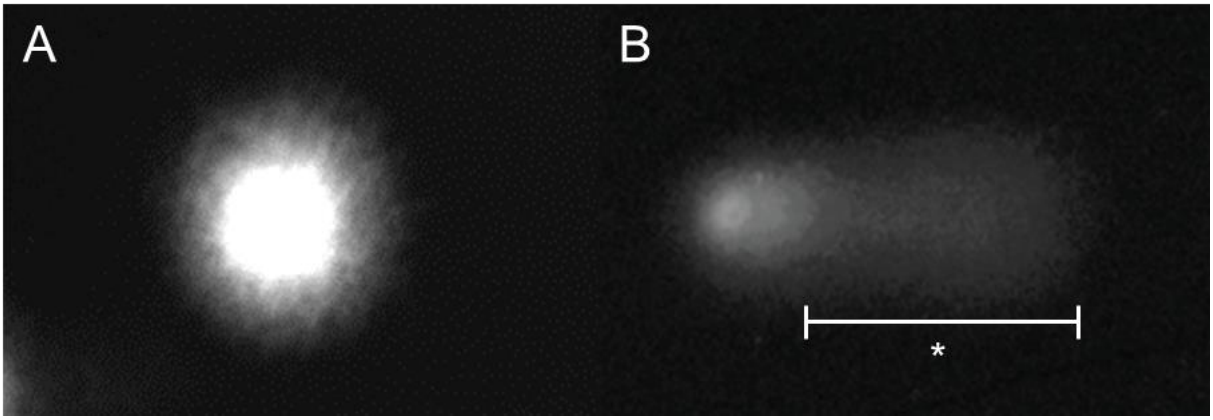
Single Cell Gel Electrophoresis (SCGE), or the comet assay, has become one of the most common methods of assessing DNA damage and repair due to its simplicity, sensitivity, and adaptability (Collins, 2004). The fundamental origins of the comet assay date back to 1976 where Cook and colleagues (Cook *et al.*, 1976), demonstrated that isolating the nucleoid was possible by lysing cells in high molarity sodium chloride and non-ionic detergents (Collins, 2004). This nucleoid comprised of the nuclear matrix of ribonucleic acid and proteins, in addition to deoxyribose nucleic acid.

Measurement of DNA damage via the comet assay can be quantified through a number of different approaches including the following:

1. Tail length – a laborious method using a photomicrograph or graticule which only allows for quantification at low levels of DNA damage.
2. Visual representation upon inspection of 100 comets.
3. Image analysis using commercially available software.
4. Automated systems

To date, image analysis software remains the most commonly used approach by researchers due to the surfeit of information provided (Collins *et al.,* 2008; Tryfidou *et al.,* 2019). A typical commercially available software will allow quantification of total intensity, tail length, % DNA in the tail, % DNA in the head, and tail moment. At very low levels of damage, tail length may be the most informative. However, generally, % DNA in the tail covers the widest range of damage and is linearly related to break frequency. Other researchers have also used tail moment as it allows for the expression of tail length and % DNA in the tail simultaneously; however, there doesn't appear to be an advantage of doing so. For one, standard dose-response curves deviate from linearity. Additionally, dependent on the software, tail moment is calculated through different algorithms, and is expressed in arbitrary units meaning comparisons across studies are difficult to interpret. Collectively, % DNA in tail is regarded as the parameter of choice due to its sensitivity (Collins *et al.,* 2008). A representation of visual image analysis is depicted in Figure 3.1.

*Figure 3.1. Depiction of lymphocyte nucleic acid head (A) and damage (B) indicated by tail under microscopy. * represents area which will be detected as tail.*



3.4.1.1 Materials and Methods

All chemicals were purchased from Sigma-Aldrich (Chemical Co., Poole, Dorset, UK). Dakin frosted slides (3"x1"; 1.2mm thick) were supplied by Richards supply Co. Ltd, London. Cover slips (22x40mm, 22x22mm; no 1 thickness) were supplied by GBH, Laboratory Supplies, Poole, England.

Upon thawing, samples were transferred to a 15ml centrifuge tube along with 3ml of PBS and centrifuged at 3500 RPM for 10 minutes. When the spin cycle was competed, the PBS was discarded, and the remaining pellet was resuspended in 3ml of PBS. Microscope slides (frosted on one end) were prepared in advance using 100µl of 1% normal melting point agarose and allowed to dry overnight. Slides were labelled in accordance to their corresponding sample. 50µl of resuspended cells were mixed with 150µl of low melting point agarose. 70µl of this combined solution was layered on top of the prepared normal melting point agarose slides (0.5%) and allowed to solidify under a 20x20 coverslip at 4°C for 5 minutes. Upon completion, the coverslip was removed and the slides were placed in a coupling jar containing the lysis solution (2.5m NaCl, 135 100mM NaEDTA, 10mM Trizma, 1% Triton-X, pH 10). The coupling jar was placed in the dark at 4°C for 1 hour. The slides were then placed in the electrophoresis tank along with electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 12.5-13) and allowed to incubate for 20 minutes in the dark. Horizontal gel electrophoresis was performed at 25V, 300 mA, 0.15 v/cm for 30 minutes at 4°C. Following electrophoresis, slides were placed into a staining jar with PBS for 10 minutes at 4°C and subsequently in double distilled water for 10 mins at 4°C; this removed any alkali and detergents from the samples. Slides were allowed to dry overnight in the dark at room

temperature. Slides were subsequently stained with 20µl SYBR ® Gold and 25ml of TE buffer (10 mM Trizma, 1 mM EDTA) for 30 minutes in the dark and analysed within 2 hours. Slides were placed under an Olympus BH-2 epifluorescence microscope (Olympus, Watford, UK). The microscope was equipped with a 100 W mercury power source, a 515-569 nm excitation filter and a 590nm barrier filter; all samples were analysed under a 40x magnification. A Hewlett Packard PC 486/33U was connected to the microscope via a Pulnix MT 765 camera. Kinetic Imaging UK (Comet, v 2.2.) provided the image processing and analysis software package, Matrox. 50 random cells were counted per each sample.

3.4.2 Electron Paramagnetic Resonance Spectroscopy

Electron Paramagnetic Resonance (EPR) spectroscopy, is a highly sensitive and accurate method for detecting free radical species such as the ascorbyl radical (Spasojevic *et al.*, 2011). This can be achieved due to free radicals containing an unpaired electron possessing a magnetic dipole from the electrons spin ($S = ½$). The energy from the spinning of the electron means the electron has two quantum spin states, and as a result have two orientations within a magnetic field (Gonzalez *et al.*, 2013); known as parallel ($ms = - ½$) and antiparallel ($ms = + ½$) orientations or Zeeman energy states. The change in energy states of the electron between these two orientations occurs when a magnetic field is applied; this is demonstrated in the equation $\Delta E = g\beta H$. Detection of free radicals is ultimately determined by the absorbance of an applied microwave frequency to the biological sample. The magnitude of the spectra recorded is directly correlated to the concentration of free radicals within a given sample.

3.4.2.1 Ascorbyl Free Radical Analysis

1ml of EDTA plasma was mixed with 1ml DMSO and gently inserted into an aqueous flat cell (ER 4110AX, AquaX cell, UK).) fixed firmly within a TM_{110} cavity of a Bruker EMX series X-band spectrometer (Bruker, U.S.A), and analysed using the following settings: frequency = 9.785 GHz; microwave power = 20 mW; modulation frequency = 100 kHz; modulation amplitude = 1.194 G. Spectral lines were collected and analysed with Data Acquisition and WinEPR software programs respectively (Version 2.11, Bruker Win EPR System). All samples were treated, processed, and analysed identically by the same investigator.

3.4.3 Serum Lipid Hydroperoxides (LOOHs)

Polyunsaturated fatty acids (PUFA) of cell membranes undergo oxidative degradation via non-enzymatic peroxidative reactions or enzymatic oxidation, resulting in the formation of lipid hydroperoxides (Elstner, 1994). LOOH's can further decompose to a number of products such as alkenals or alkanes (Gardner, 1995). The Ferrous Oxidation of Xylenol orange, or FOX reagents (FOX-I and FOX-II) are two relatively simple spectrophotometric methods for detection of LOOH *in vivo*. The protocol is based on the theory that lipid hydroperoxides oxidise ferrous (Fe^{2+}) ions to ferric (Fe^{3+}) ions; indirect quantification of LOOHs are essentially measuring the binding of ferric iron to the ferric-sensitive xylenol orange (Wolff, 1994). For the purpose of this thesis, the FOX I reagent was performed. A concentration of 0-5 $\mu M \cdot L^{-1}$ of hydrogen peroxide was used to form a standard curve, and all samples were read spectrophotometrically at an absorbance of 560 nm. To prepare the FOX I reagent, 100$\mu M.L^{-1}$ xylenol orange, 250$\mu M.L^{-1}$ ammonium ferrous sulphate, 100$\mu M.L^{-1}$ sorbitol, and 25$mM.L^{-1}$ of sulphuric acid (H_2SO_4) was combined in a volumetric flask. HPLC grade water was added until the meniscus aligned with 100ml on the flask and stored in a darkened room. 90µl of serum was pipetted into an Eppendorf tube along with 900µl of the FOX I reagent. A blank sample comprised of 90µl of HPLC-grade water and 900µl of the FOX I reagent was included and treated in the same manner. The solution was vortexed and allowed to sit in a darkened area for 30 minutes at room temperature. Upon completion, all samples were transferred to cuvettes for spectrophotometric analysis (UV mini-1240 UV-Vis Spectrophotometer). The blank sample was analysed first to determine background absorbance, followed by the experimental samples.

3.4.4 Lipid Soluble Antioxidants

HPLC was used to quantify lipid soluble antioxidants (α-tocopherol, γ-tocopherol, α-carotene, β-carotene, lycopene, retinol, and xanthophyll) within serum. Each antioxidant has a different molecular structure allowing light to be absorbed at specific absorbances (Popov & Lewin, 1996). Consequently, this absorbance provides a unique colour and retention time allowing individual antioxidants to be simultaneously quantified as per the protocol of Thurnham *et al.* (1988).

Initially, standards were formulated (containing 250 ml of; α-tocopherol, γ-tocopherol, α-carotene, β-carotene, lycopene, retinol, and xanthophyll), including 250

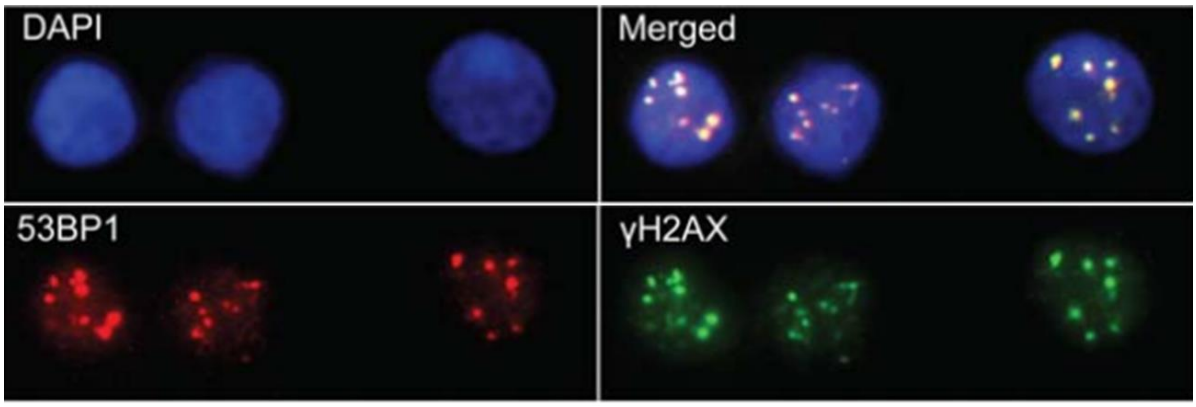
µl of an internal standard comprised of 1 g tocopherol acetate in 100 ml heptane, made up to 250 ml final volume with ethanol. The mobile phase was made by mixing methanol, acetonitrile, and dichloromethane in a ratio of 47:47:12 respectively. 300µl of plasma/serum/standard mixture was pipetted into a glass tube. Three QCs (pooled plasma) for every batch of samples run was included prior to each batch. In addition to the 250 µl of the internal standard, 500 µl of heptane was added to each tube and subsequently vortexed for 60 seconds. All sample vials were centrifuged for 5 minutes at 3000 rpm. The resulting heptane layer (350 µl) was pipetted into an identically labelled glass tube and a further 500 µl to the original sample tubes to perform a second extraction. Samples were briefly vortexed and centrifuged with the same previous parameters. The combined heptane layer (700 µl) was allowed to evaporate to dryness in a centrifugal evaporator under vacuum. Samples were reconstituted with 150 µl of mobile phase (470 ml acetonitrile, 470 ml methanol and 120 ml dichloromethane with 0.025 g of BHT), vortexed for 10 seconds and pipetted into glass inserts within numbered glass vials in the HPLC carousel. Samples were then measured using a Waters HPLC system (Waters, 717 auto sampler, Waters PDA detector, Waters 510 pump), column (Waters sunfire C18 3.5 µm, 4.6 x 100mm) and guard column (Waters Sentry Guard holder, WAT046910, waters column joining tube assay WAT084080, Waters sunfire 3.5 µm 4.6 x 20mm guard column part no.186002682). HPLC parameters were set to the following conditions; pressure 1000-2000 PSI and flow rate 1.5ml/min. Using the Waters PDA detector and Empower Analytical Software (Empower PDA software, Waters, Dublin, Ireland), samples were read across 240nm, 420nm and 550nm for the tocopherols, retinol, and the carotenes, respectively; this allowed the monitoring and adjustment of the internal standard, and quantification of the sample concentration using the external standards.

3.4.5 Dual Staining Immunohistochemistry

The most harmful DNA lesions are double-stranded breaks which can lead to severe consequences such as genomic instability. The use of DNA foci detection has previously been used in a wide range of sample types, including peripheral blood mononuclear cells, established cell lines, and cell cultures (Mariotti *et al.*, 2013; Lippi *et al.*, 2018). Upon DSB induction, the histone protein H2AX becomes phosphorylated by PI3-kinases, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia rad3-related (ATR) serine/threonine kinases at its C-terminal of the serine 139 residue

(Bonner *et al.*, 2008); known as γ-H2AX, as it was first discovered using γ-rays. Following the generation of γ-H2AX, there is a consequential activation and recruitment of downstream DNA repair signalling factors (i.e. MDC1 and 53BP1) to the site of damage. The assay is based on the premise of primary and secondary antibodies raised to the phosphorylated C-terminal of the H2AX protein (Bonner *et al.*, 2008). These can be determined using stains which identify γ-H2AX foci; in turn, this correlates with the amount of DSBs. Similarly, as the presence of foci correlates with the amount of damage, the disappearance of γ-H2AX foci is associated with DNA repair (Mariotti *et al.*, 2013). An addition of p53-binding protein 1 (53PB1) is also normally used to identify the interaction and co-localization with γ-H2AX foci (Fernandez-Capetillo *et al.*, 2003); detection of γ-H2AX and 53BP1 foci and co-localization can be observed in Figure 3.2.

Figure 3.2 Typical representative output from dual staining immunohistochemistry for γH2AX and 53BP1 foci. The merged image depicts the co-localization of γ-H2AX and 53BP.



3.4.5.1 Methods and Materials

All chemicals were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Primary Antibodies were purchased from Merck Millipore (Billerica, Massachusetts, United States). Dakin frosted slides (3"x1"; 1.2mm thick) were supplied by Richards supply Co. Ltd, London. Cover slips (22x40mm, no 1 thickness) were supplied by GBH, Laboratory Supplies, Poole, England.

3.4.5.2 H2AX Protocol

10µl of lymphocytes at a concentration of 1 x 10^6/ml were spotted onto a Superfrost plus slide, fixed by 4% formaldehyde/PBS solution for 10 minutes, and permeabilized using 0.5% Triton X-100/PBS for 10 minutes; this was followed by three washes of PBS. Slides were blocked using 1% bovine serum albumin in PBS for 1 hour. Cells were incubated in primary antibodies by dual combination of 1:1000 anti-phospho-histone γ-H2AX (Ser139, mouse) and pAB anti-53BP1 (rabbit) antibodies in 1%BSA/PBS for 1 hour. Cells were washed in 1%BSA/0.1% tween-20/PBS and incubated in 1:2000 AlexaFlour 488 conjugated antibody and 1:2000 anti-rabbit AlexaFlour 568 conjugated antibody in 1%BSA/PBS for 1 hour. Afterwards, cells were washed three times with 1%BSA/0.1% tween-20/PBS and left to dry overnight in the dark at room temperature. A coverslip was placed on all slides and mounted using VECTASHIELD containing 4',6-diamidino-2-phenylindole and analysed with an Olympus BH-2 epifluorescence microscope (Olympus, Watford, UK), using a x63 objective. Due to the absence of three-dimensional microscopy with Z-planning, the same trained individual manually focused through the whole nucleus to detect all foci in the 3D-room (Djuzenova *et al.*, 2015). A total of 100 cells were analysed per slide, providing the following parameters; percentage of cells with γ-H2AX and 53BP1 foci, mean number of γ-H2AX and 53BP1 foci per cell, and total number of γ-H2AX and 53BP1 foci. It should be noted all steps were performed in the dark and all incubation steps were performed using a darkened slide tray.

3.4.6 Superoxide Dismutase

SOD was measured spectrophotometrically using a Superoxide Dismutase Assay Kit (Cayman Chemicals, Ann Arbor, MI, USA). This specific kit operates using tetrazolium salts for the detection of SOD following the administration of xanthine oxidase.

Upon thawing, the serum with diluted with sample buffer at a ratio of 1:5. 3ml of the assay buffer was dilute using 27ml HPLC-grade water while 2ml of the sample buffer was diluted with 18ml of HPLC-grade water. The radical detector was wrapped in tin foil to protect from light. 50µl was transferred to 19.95ml of the diluted assay buffer and stable for 2 hours. 50µl of the enzyme was added to 1.95ml immediately prior to use. The SOD standard was prepared by combining 20µl of the SOD standard and 1.98ml of diluted sample buffer. Seven standards were prepared (labelled A-G) as outlined in Table 3.1.

Table 3.1. An overview of SOD standard preparations.

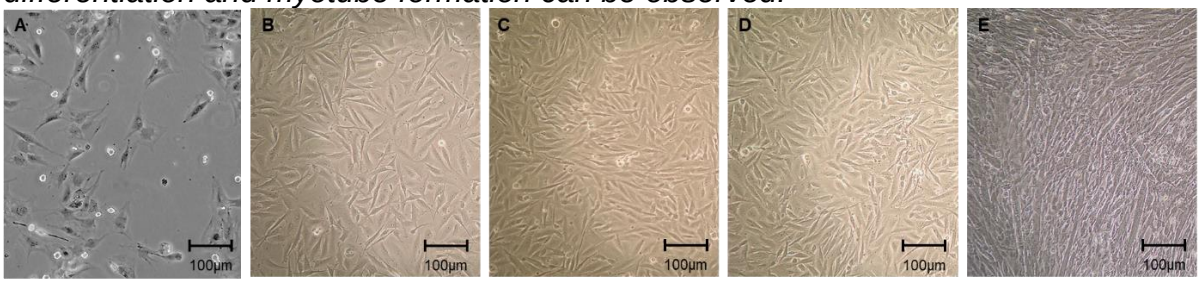
Standard	SOD (µl)	Sample Buffer (µl)	Final SOD Activity (U/ml) in Well
A	0	1,000	0
B	20	980	0.005
C	40	960	0.010
D	80	920	0.020
E	120	880	0.030
F	160	840	0.040
G	200	800	0.050

200µl of the diluted radical detector was added to the standard wells along with 10µl of SOD standard. With regards to sample wells, 10µl of sample and 200µl of radical detector was added to the specified wells. The reaction was initiated by adding 20µl of xanthine oxidase to all wells. The plate was allowed to incubate in the dark for 30 minutes at room temperature. The plate reader was set to an absorbance of 440-460 nm and analysed.

3.5 Cell Culture

Primary human lymphocyte cells and C2C12 *Mus Musculus* myoblasts (ATCC CRL-1772) were cultured in Dulbecco's Modified Eagles Medium supplemented with 10% foetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. For C2C12 differentiation, 5 x 10^4 cells were seeded and cultured in growth media until reaching 70–80% confluence (see Figure 3.3.). Media were then replaced with Dulbecco Modified Eagle Medium supplemented with 5% horse serum, 1% L-glutamine, 1% penicillin/streptomycin. Cultures were maintained at 37°C in an atmosphere of 95% humidified air and 5% CO_2. Cell viability and total cell count was assessed using the Trypan Blue Exclusion Assay in conjunction with a TC20 Automated Cell Counter (Bio-RAD, California, United States); all samples maintained above 95% viability throughout.

Figure 3.3. Time course representation of typical C2C12 Mus Musculus cells. *Note; (**A**) represents cells at day 0. (**B, C, D**) depicts cells at 80-95% confluence. (**E**) initial differentiation and myotube formation can be observed.*



3.5.1 Trypan Blue Exclusion Test of Cell Viability

The 0.4% Trypan Blue (Sigma-Aldrich, United Kingdom) exclusion test is a routine assay to determine the number of viable cells within cell suspension. Within chapter 5 this assay was used to determine cell count and cell viability pre-incubation, and following incubation for 24, 48 and 72 hours. Briefly, following isolation of lymphocytes, 10µl of cell suspension was mixed with 10µl of Trypan blue dye. The solution was added to a dual chamber counting slide and inserted into a TC20 Automated Cell Counter (Bio-RAD, California, United States). Results were expressed as total number of cells (cells/ml), total live cells (cells/ml) and percentage of live cells.

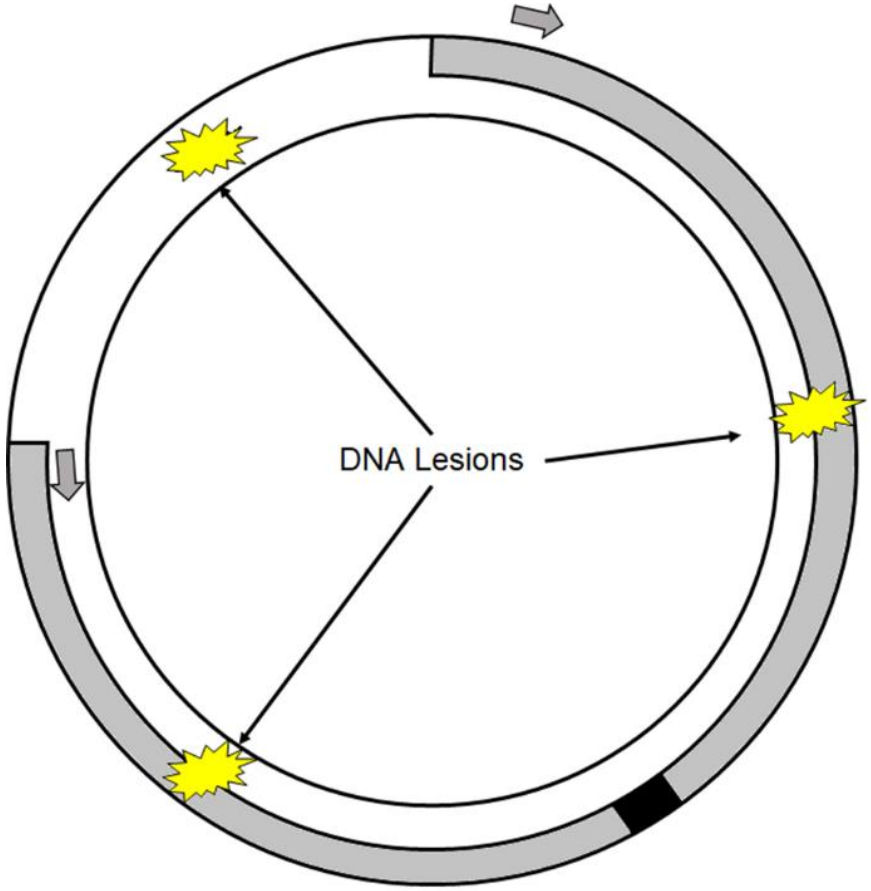
3.5.2 Culturing of Peripheral Blood Mononuclear Cells

All cell culture work was carried out in a Class II Laminar Flow Biological Safety Cabinet (Astec Microflow, Hampshire, United Kingdom). Lymphocytes were cultured within a T25 flask (Sarstedt; Numbrecht, Germany) and incubated at 37°C, 5% CO_2 for up to 72 hours. Unless stated otherwise, all chemicals mentioned in the above section were purchased from Sigma-Aldrich (Dorset, United Kingdom).

3.6 Long-Amplicon Quantitative PCR (LA-qPCR)

Recently, long-amplicon qPCR has been developed and refined for the quantification of polymerase-stalling lesions (Gonzalez-Hunt *et al.*, 2016). These large stretches of mitochondrial or nuclear DNA (10-15kb) allow for improved sensitivity and detection of various lesions (Meyer, 2010). The mechanistic underpinning theory can be observed in Figure 3.4. Cells quantified via LA-qPCR and the obtained amplification is inversely correlated with the number of DNA lesions capable of blocking or inhibiting DNA polymerase activity. According to the Poisson distribution, the quantified amplification can be compared to control samples (defined as no damage) with a subsequent relative lesion frequency calculated (Ayala-Torres *et al.*, 2000; Furda *et al.*, 2014; Furda *et al.*, 2014).

Figure 3.4. Schematic of the mitochondrial genome and the basis of LA-qPCR.



The white circle represents the genome in its entirety, while the grey section represents the target long amplicon which also coincide with the primers represented by the grey arrows. The smaller black crescent represents the small amplicon which also include primers (not shown on schematic). The lesions (represented by stars) will inhibit polymerase activity thus reducing the amplification. Amplification of the short amplicon is only inhibited by very large amounts of damage.

3.6.1 DNA Extraction from Lymphocyte and Human Muscle Cells

DNA extraction was performed using the Qiagen Genomic Tip method as detailed by (Gonzalez-Hunt *et al.*, 2016; Saunders *et al.*, 2018). Details of buffer preparation for the assay are outlined in Table 3.2.

Table 3.2. An overview of the buffers required for the Genomic Tip DNA extraction protocol and corresponding storage conditions.

Buffer	Function	Composition	Temperature
C1	Cell Lysis	1.28 M sucrose, 40 mM Tris·Cl, pH 7.5, 20 mM $MgCl_2$, 4% Triton X-100	4°C
G2	Digestion Buffer	800 mM guanidine HCL, 30 mM Tris·Cl, pH 8.0, 30 mM EDTA, pH 8.0, 5% Tween-20, 0.5% Triton X-100	2-8°C or RT
QBT	Equilibration Buffer	750 mM NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol, 0.15% Trition X-100	2-8°C or RT
QC	Wash Buffer	1 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol	2-8°C or RT
Protease/ Proteinase K	Digestion of Denatured Proteins and Proteins Bound to DNA	N/A	2-8°C when reconstituted
QF	Elution Buffer	1.25 M NaCl, 50 mM Tris·Cl, pH 8.0, 15% isopropanol	50°C

Frozen lymphocyte cells were thawed and washed in PBS for 5 minutes at 3500 RPM. The supernatant was carefully discarded, and cells were resuspended in fresh PBS to a concentration of 10^7 cells/ml. Human muscle tissue was homogenised as previous described by Saunders *et al.*, (2018). Briefly, the skeletal muscle tissue (approx. 90-150mg) was placed in 500 µl of isolation buffer I and 500 µl of isolation buffer II in a 1.5 ml Eppendorf tube, and allowed to incubate on ice for 10 minutes. Using an A and B Kimble-Chase Kontes Tissue Grinder, the tissue was homogenised until the homogenate was cloudy and free from any particulates. The final contents were centrifuged for 20 minutes at 10,000 x *g* at 4°C. The supernatant was subsequently discarded prior to applying the Genomic-Tip protocol. Following this, 0.5ml of cell suspension was added to 1 volume of ice-cold C1 buffer and 3 volumes

of ice-cold distilled water, and allowed to incubate on ice for 10 minutes in order to lyse the cells while preserving the nuclei. Cell lysate was centrifuged at 4°C for 15 minutes at 1300 g. Subsequently, 0.25ml of buffer C1 and 0.75ml of distilled water was added to nuclear pellet and vortexed to resuspend. This step was followed by a second centrifuge with the same parameters as before in order to remove all debris from the nuclear pellet. The nuclei were resuspended in 1 ml of buffer G2 and vortexed for a maximum of 30 seconds in order to lyse the nuclei, and to ensure a good flow rate through the Genomic-Tip. 25 µl of Qiagen protease was added to the solution and allowed to incubate for 30-60 minutes at 50°C. During this incubation period, the Genomic-Tip was equilibrated with 1 ml of QBT buffer and allowed to empty via gravity flow. Upon completion of protease incubation, the sample was vortexed at maximum speed for 10 seconds and promptly pipetted into the equilibrated Genomic-Tip. This was followed by a wash step comprising of 3 x 1 ml washes of buffer QC. To elute the DNA, 1 ml of the pre-warmed QF buffer was passed through the Genomic-Tip twice into a fresh 10 ml collection tube. DNA was precipitated by adding 1.4 ml of isopropanol to the eluted DNA.

3.6.2 Determination of DNA Concentration and Quality

A NanoDrop spectrophotometer (Thermo Scientific NanoDrop Technologies, Montchanin, DE, USA) was used to determine the concentration and purity of obtained DNA and RNA. Prior to adding a sample, 2 µl of a blank was placed on the sample pedestal and secured with the arm forming a ‘sample column’; the blank corresponded to the respective buffer used to elude the DNA. Concerning sample analysis, a volume of 2 µl was pipetted onto the pedestal and analysed; the pedestal was cleaned with a lint-free lab wipe prior to the blank and between every sample thereafter. The concentration and purity of DNA samples were measured at a known absorbance maxima of DNA, RNA and impurities. The $A_{260/280}$ ratio was used to determine acceptable purity, as defined by ~1.8 and ~2.0. The $A_{260/230}$ ratio was used as a secondary measure of nucleic acid purity; with 2.0-2.2 accepted as a suitable range.

3.6.3 Quantification of Template DNA

DNA samples were diluted in TE buffer to a concentration of ~10 ng/µl in a sterile 0.5 ml microcentrifuge tube. A standard curve was obtained by diluting λ/*Hind*III DNA to concentrations of 100, 50, 25, 12.5, 10, 7.5, 5.0, 2.0, and 0 ng/µl of TE buffer to produce a linear curve as outlined in Figure 3.5.

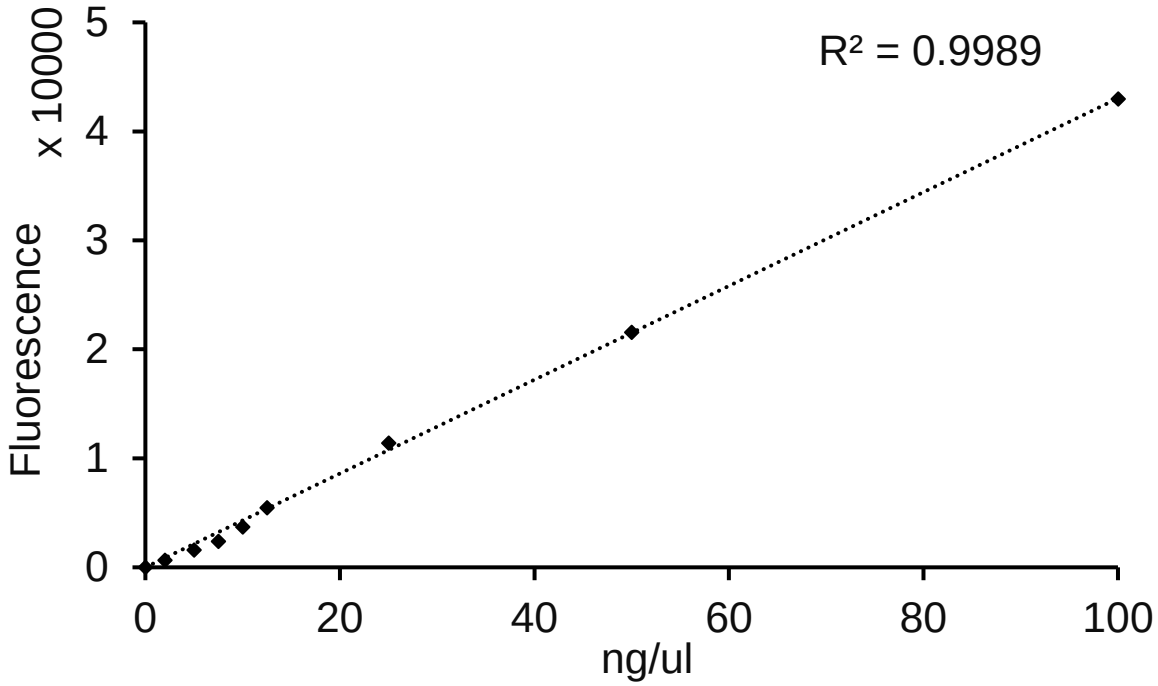


Figure 3.5 Standard curve using λ/HindIII DNA of varying concentrations.

Each of diluted λ/*Hind*III standard was added to 95 µl TE buffer to comprise a total volume of 100 µl; this was performed in triplicate wells with 5 µl of the sample DNA being added to each well. 100 µl of working PicoGreen solution (5 µl of PicoGreen per 1ml TE buffer) was added to each well and incubated in the dark for 10 minutes. Subsequently, fluorescence was measured with an excitation of 485 nm and an emission of 530 nm with a 20 second shaking step prior to reading. The quantified DNA concentrations were contrasted with the standard curve and any samples which exceeded 15ng/µl were diluted and re-measured. Samples were diluted to 3 ng/µl in TE buffer.

3.6.4 LA-qPCR Protocol

50 µl reactions were prepared by combining the following component consecutively; KAPA Long Range HotStart PCR Kit (Sigma-Aldrich, UK: Nuclease-free H2O for a final volume of 50 µl, 5 µl 3 ng/µl sample DNA [total 15 ng template], 10 µl 5× buffer solution, 1 µl 1.0 mg/ml BSA, 1 µl 10 mM dNTP mix, 2.5 µl each 10 µM primer, 3.5 µl 25 mMgCl2, 0.5 µl 2.5 U/µl KAPA HotStart polymerase). These 50µl included both a no template control and a 50% control that contained control DNA. These control samples ensured a lack of contamination in the reaction component and to ensure quantitative conditions within the linear range of fragment amplification, respectively.

Note – *Primer nucleotide sequences are outlined, and corresponding conditions are outlined in Table 3.3. The chosen primers have been previously validated in the target samples (Furda et al., 2012; Ayala-Torres et al., 2000; Gonzalez-Hunt et al., 2016).*

Table 3.3 Primers used in the analysis of human and C2C12 samples for long-amplicon PCR.

Species	Sequence	Annealing Temp	Size
Homo Sapiens	**F:** TCTAAGCCTCCTTATTCGAGCCGA **R:** TTTCATCATGCGGAGATGTTGGATGG	64°C	8.9 kb
Homo Sapiens	**F:** CCCCACAAACCCCATTACTAAACCCA **R:** TTTCATCATGCGGAGATGTTGGATGG	62°C	221 bp
M. Musculus	**F:** GCCAGCCTGACCCATAGCCATAATAT **R:** GAGAGATTTTATGGGTGTAATGCGG	64°C	10.9 kb
M. Musculus	**F:** CCCAGCTACTACCATCATTCAAGT **R:** GATGGTTTGGGAGATTGGTTGATGT	60°C	117

Each sample was vortexed briefly and subsequently centrifuged for approximately 10 seconds before being aliquoted into the well of a PCR plate at a volume of 25 µl. PCR products were amplified based on the conditions presented in Table 3.5.

Table 3.5 Thermocycler variables associated with the long- and short-mitochondrial primers.

Phase	Long Mt Primers	Short Mt Primers
Melting	94°C for 4 mins	94°C for 1 min
Amplification	26-28 cycles of melting (94°C for 15 secs)	20 cycles of melting (94°C for 15 secs)
Annealing	66°C for 12 mins	60°C for 45 sec
Final Extension	72°C for 10 minutes	72°C for 45 sec
Hold	4°C (or 8°C if being held overnight)	4°C (or 8°C if being held overnight)

To quantify products, 90 µl of TE buffer was added to 10 µl of PCR product; performed in triplicated within a 96-well PCR plate. Fluorescence was quantified via PicoGreen incubated in the dark for 10 minutes, and measured with at an excitation of 485 nm and emission of 530 nm with a 20 second shaking step prior to reading. Each of the triplicate sample values were averaged and subtracted from the no template control and fluorescence from the fluorescence values for the PCR products, including the 50% control. Based on the findings of Saunders *et al.* (2018), if the 50% control does not lie within 40-60% of the untreated controls, the data set is invalid, and refinements must be made to the cycle number for the respective primer. As per Gonzalez-Hunt *et al* (2016), each sample was analysed twice and subsequently compared through a correlation analysis; if $r^2 > 0.9$, the values were averaged for the two runs, however, if the correlation was weak, a third PCR run was performed to reduce the likelihood of outliers. A representation of this correlation can be observed in Figure 3.6.

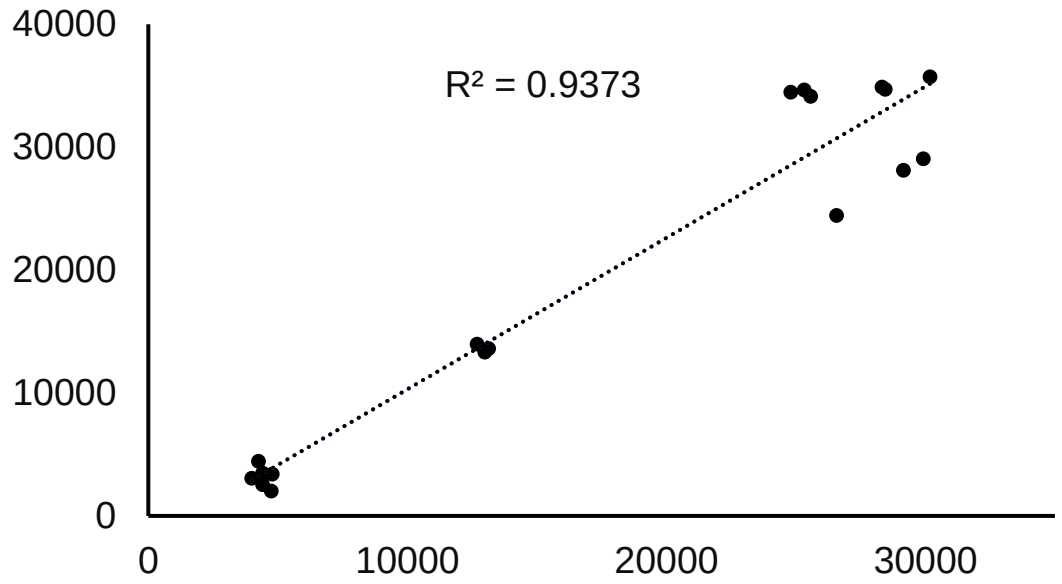


Figure 3.6 The optional quality control check as detailed by Gonzalez-Hunt et al. (2016) to ensure consistency across biological replicates.

Large mitochondrial PCR products were subsequently normalised for copy number using fluorescence values of small mitochondrial PCR products as recommended by Furda *et al.*, (2012). Before normalising samples, a correction factor was quantified by dividing each of the small mitochondrial PCR products by the mean of all the small mitochondrial products. This factor then allowed for the normalisation of large PCR products, by dividing the large mitochondrial fluorescence value by this correction factor. Subsequently, these newly quantified normalized fluorescence values were divided into each sample by the average normalized fluorescence value to give the amplification relative to the control. Finally, a negative natural log (-ln) was performed on each amplification figure to quantify the lesion frequency per fragment; this was normalised to the number of lesions/10kb; thus, quantifying the extent of mitochondrial DNA damage.

3.7 Statistical Analysis

Computerised statistical analysis was completed on all experimental data sets using SPSS statistical software (IBM, Surrey, UK, v.23).

3.7.1 Coefficient of Variance

The coefficient of variance percentage (CV%) was calculated using the following equation for each of the experimental studies:

$$CV\% = \frac{Standard\ Deviation}{Mean}\ x\ 100$$

Coefficient of variance percentages can be found within each of the experimental chapters.

3.7.2 Power Calculations

This thesis sought to examine the effects of high-intensity exercise on DNA damage as a primary outcome as measured through, but not limited to, the comet assay. The rationale for employing the use of a power calculation throughout all studies was to confidently accept or reject the null hypothesis (Batterham & Atkinson, 2005). As per the definition, the greater the statistical power, the greater the probability of obtaining a true statistical interaction effect at $P < 0.05$. The method of Cohen (1988) was employed to calculate the power of the test based on the primary outcome of DNA damage data published by Fogarty *et al.* (2011). The sample size calculation was similar to that used in other studies within this research area (Davison *et al.*, 2005; Fogarty *et al.*, 2011; Fogarty *et al.*, 2013).

3.7.3 Comparative Statistics

Upon mathematical confirmation of normal distribution via Shapiro Wilks W test, experimental data was analysed using parametric measures. The alpha level was established at $P = 0.05$ and all values are presented as mean ± standard deviation unless stated otherwise. For all experimental data sets, a two-way, repeated-measures ANOVA ascertained differences between groups (supplemented vs. placebo) and across time (pre- vs. post-exercise). Following a significant interaction effect (time *x* group), between group differences were analysed using a one-way ANOVA with a *posteriori* Tukey Honestly Significant Difference (HSD) test, while a Bonferroni paired samples t-test was used for the within time differences.

3.7.4 Effect Size

Effect sizes grant researchers further understanding of whether an intervention or experimental outcome has an effect greater than zero, or (when it is obvious that an effect exists) how big the effect is. This is important for a number of reasons: (i) it allows researchers to present the magnitude of change in a standardised metric which can be translated to practical significance, (ii) researchers can draw meta-analytic conclusions by comparing these standardised effect sizes across studies, and (iii) effect sizes can be useful in future *a priori* power analysis. The magnitude of change has been expressed as partial eta squared (effect size; ES) throughout this thesis and interpretation of these effect sizes are based on the benchmarks suggested by Cohen (1988); small (0.2), medium (0.5), and large (0.8). Partial eta squared improves the comparability of effect sizes between studies, which expresses the sum of squares of the effect in relation to the sum of squares of the effect and the sum of squares of the error associated with the effect (Keppel, 1991; Lakens, 2013).

Chapter Four

Exogenous Plant-Based Nutraceutical Supplementation and Peripheral Mononuclear Cell DNA Damage Following High Intensity Exercise

4.0 Abstract

Introduction: Plant-based nutraceuticals are categorised as nutritional supplements which contain a high concentration of antioxidants with the intention of minimising the deleterious effect of an oxidative insult. The primary aim of this novel study was to determine the effect of exogenous barley-wheat grass juice (BWJ) on indices of exercise-induced oxidative stress.

Methods: Ten (n=10) apparently healthy, recreationally trained (*V̇O2max* 55.9 ± 6 ml·kg-1min-1), males (age 22 ± 2 yrs, height 181 ± 6 cm, weight 87 ± 8 kg, BMI 27 ± 1) volunteered to participant in the study. In a randomised, double-blinded, placebo-controlled crossover design, participants consumed either a placebo, a low dose (70ml per day) of BWJ, or a high dose (140ml per day) of BWJ for 7-days. Experimental exercise consisted of a standard maximal oxygen uptake test until volitional fatigue.

Results: DNA damage increased following exercise across all groups (time x group; $p<0.05$, ES = 0.7), although there was no selective difference for intervention ($p>0,05$). There was a main effect for time in lipid hydroperoxide concentration (pooled-group data, pre- vs. post-exercise, $p<0.05$, ES = 0.2) demonstrating that exercise increased lipid peroxidation. Superoxide dismutase activity (SOD) increased by 44.7% following BWJ supplementation (pooled group data, pre- vs. post). The ascorbyl free radical ($p<0.05$, ES = 0.26), α-tocopherol ($p=0.007$, ES = 0.2), and xanthophyll ($p=0.000$, ES = 0.5), increased between the pre- and post-exercise time points indicating a main effect of time.

Conclusions: This study illustrates that a 7-day supplementation period of a novel plant-derived nutraceutical product is insufficient at attenuating exercise-induced oxidative damage. It is possible that with a larger sample size, and longer supplementation period, this novel plant-based nutraceutical could potentially offer effective prophylaxis against exercise-induced oxidative stress; as such, this justifies the need for further research.

4.1 Introduction

The physiological generation of RONS is an integral part of the biological redox equilibrium, and they play a salient regulatory role in cell signalling (Elokda & Nielsen, 2007). When the accumulation of RONS exceeds the endogenous antioxidant defence system, they incite an oxidative insult to important biological molecules, such as nucleic acids and lipids (Radak *et al.*, 2013). Consequently, RONS have been implicated in pathological diseases, including cancer and diabetes (Alfadda & Sallam, 2012). Moreover, the chronic, detrimental accumulation of RONS activates stress-sensitive intracellular pathways that generate downstream epigenetic modifications, resulting in cell damage and apoptosis (Sweazea *et al.*, 2017).

Exercise-associated sources of RONS include mitochondrial oxidative leakage, NADPH oxidase and inflammatory processes (Ji, 1996; Jackson, 2000), and there is a plethora of data demonstrating that exercise of increasing intensity and/or duration exacerbates the accumulation of RONS (Davison *et al.*, 2005; Silva & Lima, 2014). Consequently, this alters the redox homeostasis towards a more pro-oxidant state, and in essence, oxidative stress must be controlled. Fortunately, the body possesses an elaborate endogenous defence system comprised of enzymatic and non-enzymatic antioxidants; including superoxide dismutase (SOD), catalase (CAT), ascorbic acid, and α-tocopherol (Birben *et al.*, 2012). These antioxidants contribute to the protection of redox disturbances within the cell, alongside preventing against RONS toxicity (Bouayed & Bohn, 2010).

Additionally, there are numerous dietary antioxidants that can be consumed which contribute to an enhanced cellular protection. Ascorbic acid for example, effectively scavenges RONS and resynthesises α-tocopherol (Sasazuki *et al.*, 2008). Equally, during lipid peroxidation, α-tocopherol acts as a reducing agent to peroxyl free radicals; thus, inhibiting further propagation (Niki *et al.*, 2014). Indeed, there is extensive research demonstrating that antioxidant supplementation attenuates exercise-induced oxidative stress (Keong *et al.*, 2006; Taghiyar *et al.*, 2013). More recently, phytochemicals from plant-derived nutraceuticals have been utilised in animal studies investigating oxidative damage and the aging process (Morillas-Ruiz *et al.*, 2006; Leenders *et al.*, 2014; De Morais Cardoso *et al.*, 2017; Saad *et al.*, 2017), and Sweazea *et al.* (2017) has shown a significant elevation in plasma catalase concentration, following oral administration of a plant-based nutraceutical. Research would suggest plant-based diets to be superior from a health perspective (Tachon *et*

al., 2013; Casas *et al.*, 2014). Indeed, recent evidence has indicated consumption of whole plant-based dietary patterns to be linked to a myriad of beneficial health benefits including reducing obesity risk factors, lowering elevated LDL-cholesterol, and decreasing the potential of pathological diseases such as cardiovascular disease, coronary heart disease, and several cancers (Slavin & Lloyd, 2012; Miller *et al.*, 2017; Dreher, 2018a; Veronese *et al.*, 2018; Dreher, 2018b). Considering the link between disease, and the role of RONS, work has focused on the role plant-based food produce, and/or supplementation, on the generation and consequences of RONS generation.

The combination of barley- (*Hordeum vulgare L.*) and wheat-grass (*Triticum aestivum*) is a novel oral plant-based nutraceutical, however, research regarding their efficacy is absent, particularly in the context of exercise and oxidative stress. Barley grass is often cited as a functional food due to its nutrient dense, and health promoting properties; for example, gamma-aminobutyric acid, flavonoids, superoxide dismutase, catalase, chlorophyll, alpha-tocopherol, ascorbic acid, saponarin, and polyphenols, are all found in significant quantities (Zeng *et al.*, 2018). Due to the abundant nutritional properties of barley grass, it processes a number of health promoting effects including promoting sleep (Grandner *et al.*, 2014; Zeng *et al.*, 2015), antidiabetic properties (Yu *et al.*, 2002; Lyer & Venugopal, 2010; Yu *et al.*, 2012), blood pressure regulation (Richter *et al.*, 2010; Lahouar *et al.*, 2015), enhanced immunity (Moza & Gujral, 2016; Kim *et al.*, 2017), and anti-inflammatory effects (Cremer *et al.*, 1996; Ferrone *et al.*, 2007; Seo *et al.*, 2014). In addition, due to the plethora of antioxidants within barley grass (including, *γ*-tocopherol, glutathione, succinate, superoxide dismutase, 2″-*O*-glycosyl isovitexin, protoheme, lutonarin, saponarin, isoorientin, and orientin (Kitta *et al.*, 1991; Osawa *et al.*, 1992; Lee *et al.*, 1994; Choe *et al.*, 2010; Templer *et al.*, 2017), a reasonable hypothesise could be that consumption of barley grass may reduce detrimental effects of oxidative stress. Ghavami and colleagues (2014) illustrated that oral barley grass supplementation reduced radiation-induced DNA damage as measured by the comet assay. These finding are also supported by *in vitro* hydrogen peroxide administration, and the free radical scavenging effect of barley grass (Lee *et al.*, 2003). Likewise, wheatgrass contains an abundant source of ascorbic acid, α-tocopherol, and SOD which can attenuate the accumulation of RONS (Kulkami *et al.*, 2006). Similarly to barley grass, there are a myriad of health benefits associated with wheatgrass consumption including hepatoprotective roles, anti-hyperlipidemic,

hypoglycemic effect, and anti-inflammatory (Kothari *et al.*, 2008; Grunewald, 2009; Arya & Kumar, 2011). Furthermore, Sethi *et al.* (2010) observed a reduction in lipid peroxidation, and a restoration of enzymatic antioxidant concentration following wheatgrass supplementation. In addition, wheatgrass supplementation has also been shown to enhance the antioxidant defence system and simultaneously improve blood sugar of diabetic rats (Shakya *et al.*, 2012; Mis *et al.*, 2018).

To date, no study has quantified the efficacy of supplementing with a wheat-barley grass combination on oxidative damage following exhaustive exercise. The primary aim of this study was to determine the efficacy of wheat and barley grass ingestion against intracellular DNA damage and lipid peroxidation following a bout of maximal, exhaustive treadmill exercise.

4.2 Materials and Methods

4.2.1 Participants

Participants were recreationally active males, and all completed a medical history questionnaire (Appendix E), along with providing written informed consent, prior to commencing the study. All participants were non-smokers, and free from any form of medication or antioxidant supplementation for 4 weeks prior to, and throughout the study. All participants were fasted for 12-hrs before experimental testing as this was necessary to standardize inter-participant blood biochemistry (Simundic *et al.*, 2013). Participants could drink water *ad libitum*. The study was conducted in accordance with the Declaration of Helsinki and approved by a local University Ethics Committee (REC/15/0092; See Appendix A)

4.2.2. Exercise Habituation

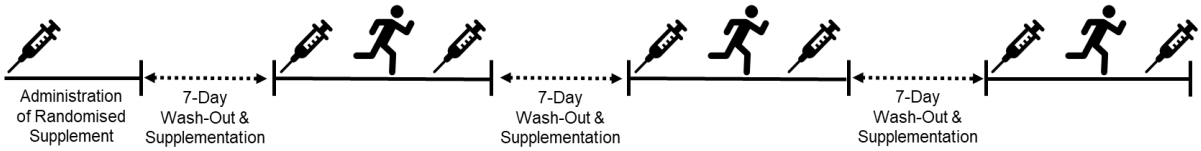
Prior to experimental testing, participants were instructed to attend the laboratory for preliminary baseline and anthropometric testing (Table 4.1). Following this, participants exercised for 10 min at 50% of maximum heart rate on a motorised treadmill (HP Comos, Hamburg, Germany) for the sole purpose of familiarisation.

4.2.3. Study Design and Nutraceutical Preparation

A randomised, double-blinded, placebo-controlled, crossover design was employed. Participants were allocated to one of three groups using simple randomisation as outlined by Suresh (2011) via a computer-simulated model. Participants allocated to

the placebo (*n* = 10) group consumed 70 mL per day of a liquid-form placebo. Participants assigned to the low-dose (*n* = 10) and high-dose (*n* = 10) group consumed 70 mL or 140 mL per day of a fresh plant-based nutraceutical (BWJ: Tiro Nutrition; Northern Ireland, UK), respectively. The supplementation phase lasted 7-days, followed by a 7-day wash-out period before commencing the next phase of supplementation (Boorsma *et al.*, 2014). An overview of the experimental design is outlined in Figure 4.1. The fresh barley-wheat grass juice (BWJ) was prepared as follows: the young shoots of barley (*Hordeum vulgare L.*) and wheat (*Triticum aestivum*) grass were grown indoors and a single true leaf was harvested 10–14 days following gemination; leaves were rinsed and cold-pressed to extract the juice using a slow speed twin auger screw press. A juice-blend of 50:50 from barley-wheat grass was combined with 1–2% volume of lemon juice; the final product was bottled into polyethylene terephthalate bottles and treated by high pressure pasteurization. For the purpose of this study all supplementation was consumed within 1-2 weeks from pressing.

Figure 4.1 Schematic overview of the experimental protocol.



Participants were allocated to one of the supplemental groups for 7 days. Following a 7-day washout period, participants crossed to another supplemental phase; this was repeated so each participant experienced each supplemented group. Participants completed a $\dot{V}O_{2max}$ (maximum oxygen uptake) test a total of 3 times throughout the study duration.

4.2.4. Experimental Testing

Participants abstained from exercise and alcohol consumption for 48-hrs before completing a standardized maximal oxygen uptake test. To negate the possibility of a diurnal variation effect on blood indices, all participants attended the laboratory at the same time (08:00) on each experimental day. Briefly, a motorized treadmill was set at 11 km/h with a 1% gradient rise at each 1-min interval until volitional fatigue. Validation of $\dot{V}O_{2max}$ was confirmed when the respiratory exchange ratio was above 1.15 arbitrary units, a plateau in the oxygen uptake/exercise intensity relationship (>2 mL kg^{-1} min^{-1}),

and a heart rate within 10 beats min^{-1} of age-predicted maximum. All participants across each of the experimental phases achieved $\dot{V}O_{2max}$.

4.2.5. Biochemical Indices

Blood was extracted from a prominent antecubital forearm vein before supplementation, and then again at the pre- and post-exercise time points. All blood was centrifuged, aliquoted, and stored at −80 °C prior to biochemical analysis.

An exercise-induced haemoconcentration was determined using the equations of Dill and Costill (1974), incorporating haemoglobin and haematocrit indices; this was used to account for acute-exercise induced plasma volume changes. Packed cell volume (%) was measured using the microcapillary reader technique, and corrected by 1.5% for plasma trapped within erythrocytes (Dacie & Lewis, 1968).

4.2.6. Deoxyribonucleic Acid (DNA)

DNA damage was measured in human peripheral blood mononuclear cells (PBMCs) using the comet assay as detailed by Singh *et al.* (1988). Full assay description is found in section 3.4.1.1. The intra/inter-assay coefficient of variation (CV's) was <8%.

4.2.7. Lipid Hydroperoxides (LOOH)

Serum LOOH was measured spectrophotometrically using the method of Wolff (1994) as per section 3.4.3. The intra/inter-assay coefficient of variation (CV's) was <5%.

4.2.8. Lipid Soluble Antioxidants (LSA)

LSA were analysed by simultaneous determination using the high-performance liquid chromatography (HPLC) method as described by Thurnham *et al.* (1988) as per section 3.4.4. The intra/inter-assay coefficient of variation (CV's) was <7%.

4.2.9. Electron Paramagnetic Resonance (EPR) Spectroscopy

The ascorbyl free radical was measured using EPR on a Bruker EMX spectrometer (Bruker Instruments Inc., Billerica, MA, USA) as per section 3.4.2.1. The Intra/inter assay CV were <5 and <6% respectively.

4.2.10. Superoxide Dismutase (SOD)

Extracellular SOD activity was measured spectrophotometrically using a Superoxide Dismutase Assay Kit (Cayman Chemicals, Ann Arbor, MI, USA) as outlined in section 3.4.6. The intra-assay coefficient of variation was 3%.

4.2.11. Statistical Analysis

The method of Cohen was utilized to calculate the prospective power of the test based on DNA damage data published by Fogarty *et al.* (2011). SPSS statistical software (IBM, Surrey, UK, v.23) was used to analyse data sets, and data normality was determined using the Shapiro-Wilks test ($P > 0.05$). A two-way, repeated-measures ANOVA ascertained differences between groups and across time. Following a significant interaction effect (time × group, $P < 0.05$), between group differences were subsequently analysed using a one-way ANOVA, while a Bonferroni paired samples *t*-test was used for within time differences. All significant changes were established at $P < 0.05$. The magnitude of change is expressed as partial eta squared (effect size, ES) throughout.

4.3. Results

4.3.1. Baseline Data and Compliance

Participant characteristic data and performance variables during each of the supplemental phases are presented in Table 4.1. There were no differences at baseline for any of these parameters ($P > 0.05$). All ten participants (100%) completed all three arms of the crossover trial. From a total of 280 possible supplementation opportunities, there was a 99% compliance of consumption as one participant experienced emesis on two occasions following consumption of BWJ during the high-dose phase. No other adverse side effects as a result of consumption were reported during the supplementation period.

Table 4.1. Participant characteristics (n = 10).

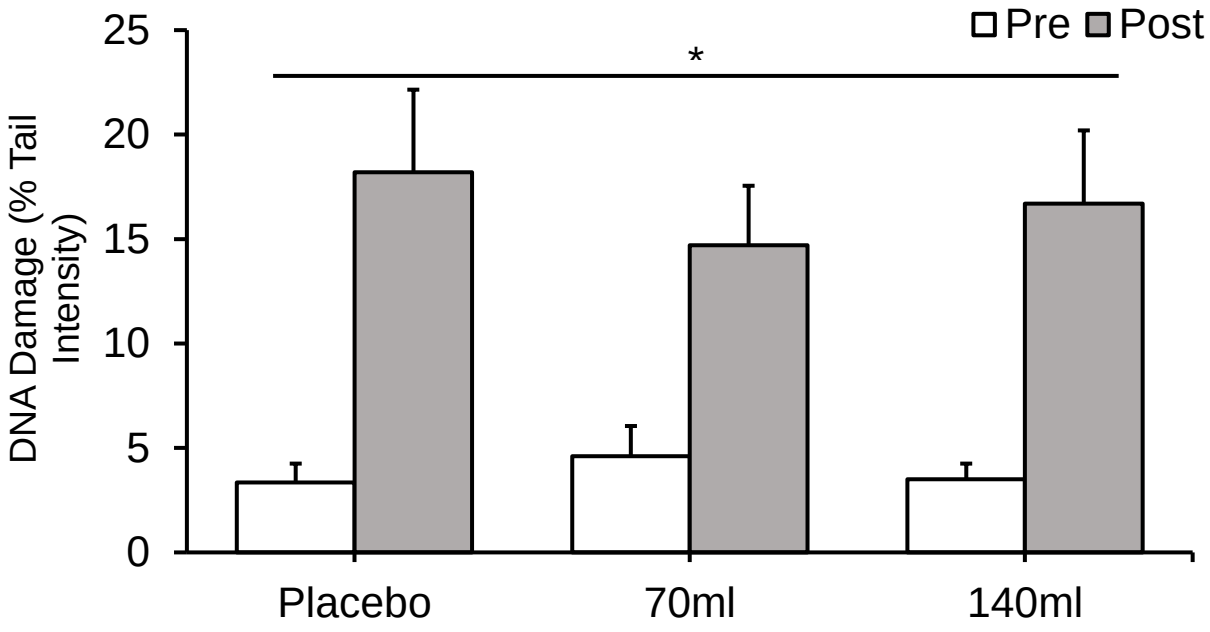
Participant Characteristics	**Baseline Data**		
Age (yrs)	21.5 ± 2		
Height (cm)	180.5 ± 6		
Weight (kg)	86.6 ± 8		
Resting HR (bpm)	69.5 ± 5		
Exercise Characteristics	**Placebo**	**Low**	**High**
Maximum HR (bpm)	189 ± 8	187 ± 8	189 ± 8
$\dot{V}O_{2max}$ (mL kg^{-1} min^{-1})	55.1 ± 6	57.2 ± 5	55.4 ± 7
TTE (mins)	9.3 ± 2	8.4 ± 2	8.5 ± 2

All values are expressed as Mean ± Standard Deviation. Abbreviations: cm—centimetres; kg = kilograms; yrs—years; HR = heart rate; bpm = beats per minute, $\dot{V}O_{2max}$—maximum oxygen uptake; TTE = time to exhaustion; mins = minutes.

4.3.2. DNA Damage

Data is presented as % tail DNA, where an increase in % tail DNA demonstrates an increase in DNA damage (Figure 4.2). There was an interaction effect for time × group ($P < 0.05$, ES = 0.6), and the post-hoc analysis indicated a difference between pre- and post-exercise for each of the three groups ($P < 0.05$, ES = 0.7). There was also a main effect for time (pooled group pre- vs. post-exercise, $P < 0.05$, ES = 0.7). Collectively this data demonstrates that exercise increased DNA damage. The increase in DNA damage as a function of exercise *per se* was Δ (delta change is expressed as the percentage change from pre- to post-exercise) 18.2%, Δ9% and Δ13% for placebo, low-dose and high-dose BWJ groups respectively, suggesting that the barley- wheat-grass juice partially attenuated the rise in DNA damage compared with the placebo; however, these changes were not significant ($P > 0.05$).

Figure 4.2. DNA damage expressed as Tail % intensity at pre- and post-exercise across groups (n = 10).

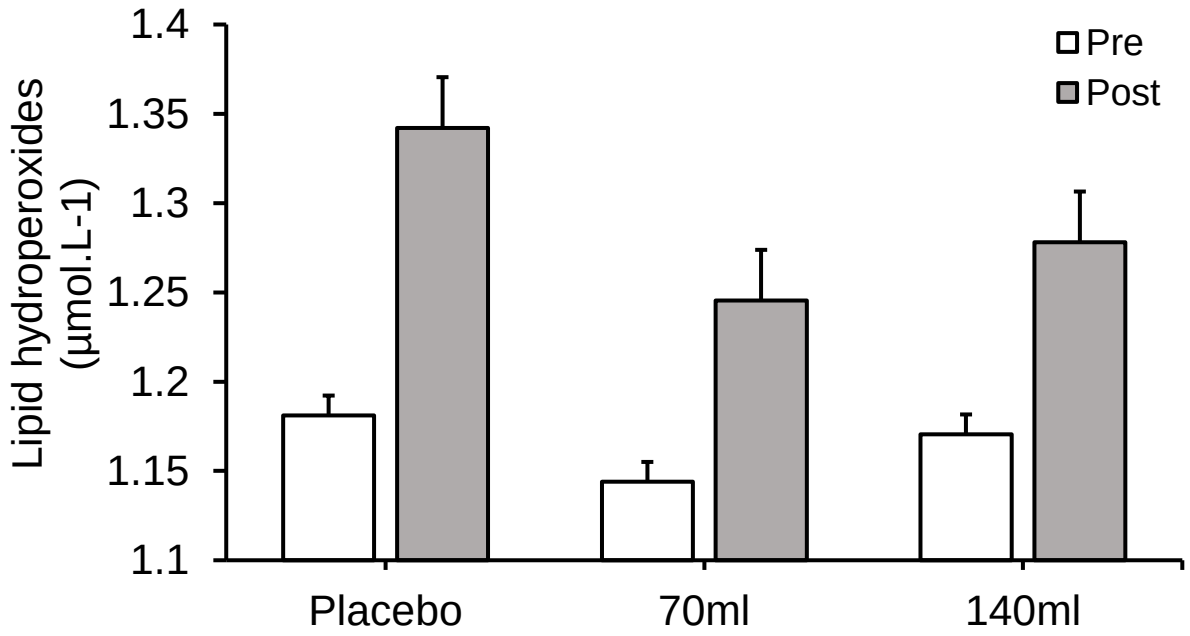


Data expressed as a mean ± standard deviation. represents a significant interaction effect (P < 0.05) within group.*

4.3.3. Lipid Hydroperoxide

There was no time × group interaction ($P > 0.05$) as observed in Figure 4.3; however, there was a main effect for time (pooled group pre- vs. post-exercise, $P < 0.05$, ES = 0.26), i.e., exercise increased lipid peroxidation. Within individual groups, lipid hydroperoxides increased by Δ12.6%, Δ7.3% and Δ7.8% for placebo, low- and high-dose BWJ groups respectively, suggesting that the supplementation partially attenuated the rise in lipid hydroperoxides compared with placebo (although this was not significant as a function of time × group).

Figure 4.3. Lipid hydroperoxides (mean ± SD) at pre- and post-exercise across groups (n = 10).



4.3.4. Lipid Soluble Antioxidants

Lipid soluble antioxidant (LSA) concentration as observed in Table 4.2 demonstrate an interaction effect of time × group for γ-tocopherol (P = 0.03, ES = 0.03, Δ27.1%) within the placebo group; however, there was no significant interaction effect between supplemental groups and time on any of the other LSA, (P > 0.05). A main effect of time (pooled data) showed an increase (P < 0.05) between baseline and post-exercise time points for α-tocopherol (Δ10.5%), γ-tocopherol (Δ23.5%), and xanthophyll (Δ64.2%) concentrations. The main effect of time (pooled date) also demonstrated an increase in α-tocopherol (P = 0.007, ES = 0.2, Δ7.9%) and xanthophyll (P = 0.000, ES = 0.51, Δ14.9%) between pre- and post-exercise.

Table 4.2. Lipid soluble antioxidants at rest (pre-exercise) and following exhaustive exercise for placebo, low-dose and high-dose supplemented groups.

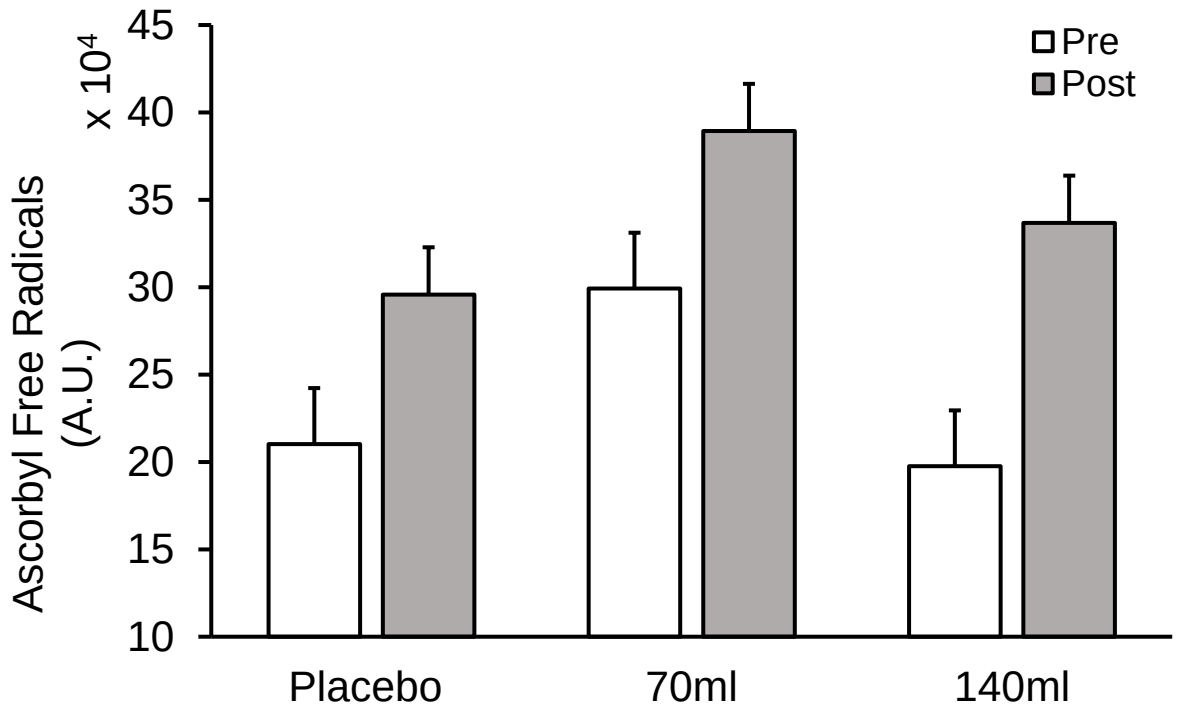
Lipid Soluble Antioxidants	Baseline	Rest	Exercise	Δ% (B-R)	Δ% (R-E)
α-Tocopherol					
Placebo	20.90 ± 1.7	21.17 ± 4.1	23.76 ± 4.3	1.3	12.2 #
Low	20.90 ± 1.7	21.71 ± 3.5	22.97 ± 3.8	3.9	5.8 #
High	20.90 ± 1.7	21.30 ± 3.8	22.53 ± 3.5	1.9	5.8 #
γ-Tocopherol					
Placebo	1.19 ± 0.4	1.44 ± 0.1	1.83 ± 0.7	21.0	27.1 *
Low	1.19 ± 0.4	1.25 ± 0.4	1.17 ± 0.3	5.0	−6.4
High	1.19 ± 0.4	1.44 ± 0.5	1.41 ± 0.5	21.0	−2.1
α-Carotene					
Placebo	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0	0
Low	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	−20.0	0
High	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0	−20.0
Retinol					
Placebo	1.96 ± 0.4	1.94 ± 0.5	2.20 ± 0.5	−1.0	13.4
Low	1.96 ± 0.4	1.94 ± 0.4	2.14 ± 0.6	−1.0	10.3
High	1.96 ± 0.4	2.00 ± 0.5	2.32 ± 0.3	2.0	16.0
Xanthophyll					
Placebo	0.28 ± 0.1	0.30 ± 0.1	0.37 ± 0.1	7.1	23.3 #
Low	0.28 ± 0.1	0.50 ± 0.3	0.54 ± 0.3	78.6 #	8.0 #
High	0.28 ± 0.1	0.45 ± 0.1	0.51 ± 0.2	60.7 #	13.3 #

*All values are expressed as means ± standard deviation and expressed as mmol·L^{-1}. B = Baseline; R = Rest; E = Exercise. * denotes significant interaction effects of group and time (P < 0.05). # denotes significant main effects of time at the pre vs. post exercise time points.*

4.3.5. Ascorbyl Free Radicals

There were no within or between groups differences as shown in Figure 4.4. ($P > 0.05$), however, there was a main effect for time (pooled group pre- vs. post-exercise, $p < 0.05$, ES = 0.26, Δ44.5%) demonstrating that exercise increased ascorbyl free radical concentration.

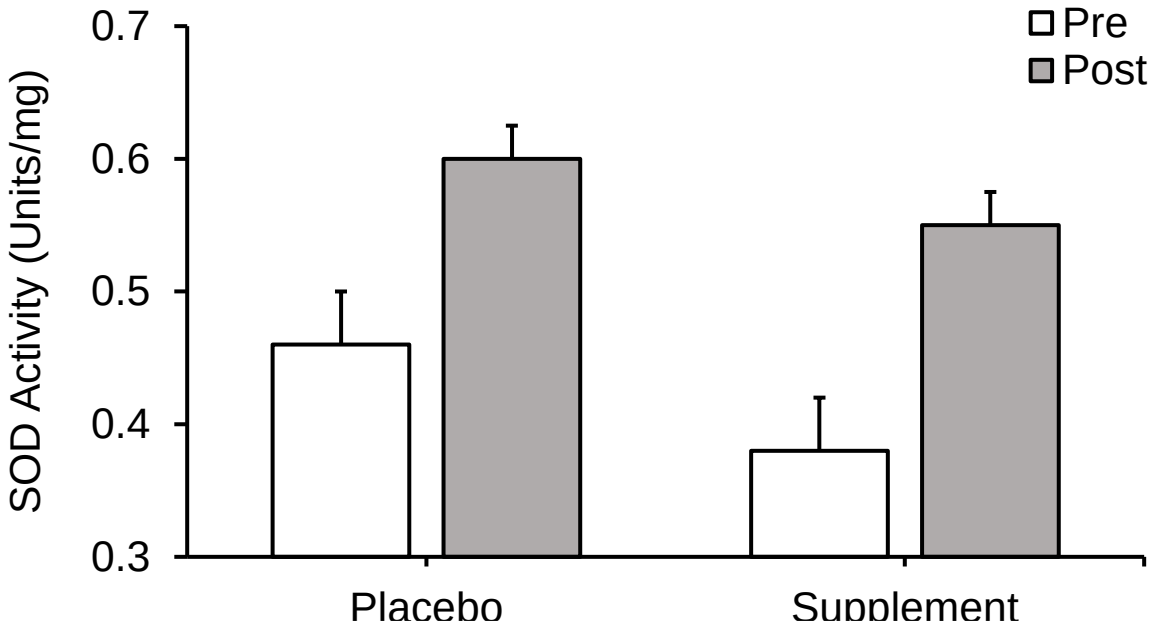
Figure 4.4. Ascorbyl free radical concentration (mean ± SD) at pre- and post-exercise across groups (n = 10).



4.3.6. Superoxide Dismutase

Although there was no interaction effect (group × time, $P > 0.05$), SOD increased by Δ 44.7% in the supplemented group (pooled supplement data) and Δ 30% in the placebo group following a single bout of exhaustive exercise (Figure 4.5). That said, there was a main effect observed for both group and time (pooled data, $P < 0.05$).

Figure 4.5. Superoxide dismutase activity (SOD) (mean ± SD) at pre- and post-exercise across groups (n = 10).



Time points comparing placebo to pooled barley grass groups. Values expressed as mean and standard deviation.

4.4 Discussion

The primary aim of this study was to determine the antioxidant effect of a novel plant-based nutraceutical supplement on exercise-induced oxidative damage. This study demonstrates that exhaustive, high-intensity exercise leads to DNA damage and lipid peroxidation, and it is conceivable these perturbations may disrupt normal biological function. There was no clear antioxidant effect with regards to selectively attenuating DNA and lipid damage following exercise. That said, the novel BWJ supplement as observed, and expressed as a delta change, seems to marginally attenuate oxidative damage (although not from a statistical perspective), and further work is merited to fully explore this relationship.

4.4.1. DNA Damage

Previous research has established that exhaustive exercise is sufficient to induce oxidative modification to DNA and lipids by increasing the generation of damaging free radicals; specifically, the hydroxyl radical (Keong *et al.*, 2006; Lambertucci *et al.*, 2007). The present study corroborates this supposition by demonstrating that DNA damage as measured by the comet assay, increases following maximal exercise ($P < 0.05$, ES = 0.73, Δ13.4%). The comet assay is a reliable and sensitive technique for detecting DNA strand breaks (Collins *et al.*, 2008), and has been utilised within exercise-related research (Davison *et al.*, 2016).

RONS-associated with exercise, are generated through several sources including mitochondrial oxidative phosphorylation, and enzymatic complexes such as NADPH oxidase and xanthine oxidase (Jackson *et al.*, 2016). Studies have demonstrated that superoxide is produced from NADH dehydrogenase and cytochrome c oxidoreductase within the electron transport chain (Urso & Clarkson, 2003). Mitochondrial RONS generation is a by-product of normal metabolism and as a result, the persistent nature of oxidative phosphorylation renders mitochondria one of the primary sources of endogenous RONS (Andreyev *et al.*, 2015). As indicated previously, it should be noted that this mechanism is somewhat controversial, and research suggests that superoxide generation from the electron transport chain is greater at rest when compared to exercise (Saint-Pierre *et al.*, 2002; Goncalves *et al.*, 2015). As suggested by Austin *et al.* (2011), it is also possible that during exercise, the increase in mitochondrial respiration causes an increase in hydrogen peroxide via peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α). Paradoxically, as mitochondrial activity

increases, PGC-1α regulates the expression of enzymatic antioxidants such as SOD and CAT (Saint-Pierre *et al.*, 2006). As a final point, it is highly probably that the observed increase in oxidative DNA damage is a partial consequence of myofibril damage from the exhaustive exercise. In turn, the activation of phagocytes, and potentially endothelial xanthine oxidase, may enhance the production of RONS; resulting in peripheral leukocyte damage (Tsai *et al.*, 2001). Excessive concentrations of superoxide can promote the formation of other reactive species which directly damage DNA and lipids (Guetens & De Boeck, 2002).

The data from this study, which indicates a single bout of exercise is sufficient to induce DNA damage, concurs in line with previously published literature (Zhang *et al.*, 2004; Keong *et al.*, 2006; Taghiyar *et al.*, 2013). It is proposed that this exercise-induced DNA modification (albeit single stranded DNA damage) may perhaps be beneficial for downstream cell signalling. It is conventionally accepted that the chronic accumulation of RONS is implicated in pathological conditions as a consequence of oxidatively modified DNA base lesions (Bo *et al.*, 2013). Specifically, the lesion 7,8,-dihydro-8-oxoguanine (8-oxoG), and subsequent product 2,6-diamino-4-hydroxy-5-formamido-pyrimidine, is most abundantly generated due its low redox potential (Langmaier *et al.*, 2004). For one, baseline 8oxo-G are significantly elevated in cancer patients, when compared to healthy individuals (Roskowski *et al.*, 2011). The removal of these lesions is primarily repaired through the base excision repair (BER) pathway, via 8-oxoguanine DNA glycosylase1 (OGG1) (Lindahl & Barnes, 2000). Recent evidence demonstrates that the knockout of OGG1 leads to the supra-physiological accumulation of 8-oxoG; subsequently leading to anomalous immune responses and metabolic disorders (Pan *et al.*, 2016). Additionally, activation of OGG1 causes the downstream expression of specific transcription factors as a function of an oxidative insult (Nishiyama *et al.*, 1994). Recently, Pan and colleagues (Pan *et al.*, 2016), have elucidated the complex relationship between 8-oxoG, OGG1, and the NF-κB-driven gene-expression pathway. Although the mechanisms are not currently well understood, it is clear that DNA base lesions, and their cognate repair enzymes, play critical roles in signal-transduction pathways. This highlights the inherent need to investigate whether antioxidant supplementation is beneficial to these repair pathways, or if they hinder signal-transduction regulation and downstream repair processes; this can be observed in the data examining antioxidant supplementation and the blunting of the beneficial adaptations of exercise (Gomez-Cabrera *et al.*, 2005; Wadley *et al.*, 2013; Merry &

Ristow, 2016). It should be noted however, that the DNA changes quantified, and observed in our experiment is single-stranded damage, and not base oxidation *per se*, and future work should focus on DNA-BER repair pathways and mechanisms, to provide context to the notion that BER is activated following exercise-induced DNA oxidation.

4.4.2. Lipid Peroxidation

From the data of the present study, lipid hydroperoxides increased following exercise (Δ 9.2%), but there was no selective difference as a function of supplementation. Previously published data demonstrates that exercise can exacerbate lipid hydroperoxides (McClean *et al.*, 2015) which can subsequently damage DNA (Marnett, 2002).

Lipid hydroperoxides *per se* are by-products of the lipid peroxidation cascade pathway, and they form via hydrogen abstraction from a polyunsaturated fatty acid side chain (Davison *et al.*, 2005; Halliwell & Gutteridge, 2007). Oxidation of vascular lipid-membranes can generate lipid hydroperoxides through the exercise-induced production of molecular free radical species (Taghiyar *et al.*, 2013). This decomposition of polyunsaturated fatty acids can produce an array of mutagenic compounds, and damaging intermediates, including alkoxyl free radicals (Davison *et al.*, 2008), and malondialdehyde (Shadab *et al.*, 2014); both molecules are capable of directly damaging DNA, whilst alkoxyl free radicals may perpetuate lipid peroxidation (Davison *et al.*, 2008). These downstream products can modify membrane fluidity and permeability, thus increasing the susceptibility of free radical damage to DNA (Fogarty *et al.*, 2011). In addition to this hydroxyl radical attack, it is also entirely plausible that during maximal exercise, there is a reduced binding capacity of iron from transferrin; thus, increasing the opportunity for the generation of hydroxyl radicals via a Fenton-related mechanism (Burgos *et al.*, 2018).

4.4.3. Prophylactic Effect of Plant-Derived Nutraceuticals

Research ascertains that barley- and wheat-grass contain enzymatic antioxidants such as SOD (Bamforth, 1983; Markham & Mitchell, 2003), glutathione peroxidase (GPx), and CAT (Janda *et al.*, 2003). Previous work has also identified a significant concentration of ascorbic acid and α-tocopherol within young shoots of barley- and wheat-grass (Nishiyama *et al.*, 1994; Paulickova *et al.*, 2007). Moreover, the identification of these

antioxidants, and following barley grass supplementation, oxidative stress is reduced in type 2 diabetes mellitus (Yu *et al.*, 2002).

Although a marginal attenuation in DNA damage and lipid peroxidation following supplementation was observed, there was no difference between supplementation and any parameter of exercise-induced oxidative stress. Given the novel nature of the nutraceutical, it is difficult to ascertain the specific active antioxidants without the use of ultra-high-performance lipid chromatography-mass spectrometry (UHPLC-MS). While this study provides pioneering data, quantification of enzymatic and non-enzymatic antioxidants via UHPLC-MS would offer invaluable guidance regarding an accurate supplementation dose, and length of supplementation period. Previous research by Yi *et al.* (2011) demonstrated a reduction in urinary 8-OHdG and MDA following supplementation of 100 mL of wheatgrass for 14 days. Additionally, Shyam *et al.*, (2007) observed a reduction in blood MDA and reduced glutathione, and an increase in total antioxidant status, superoxide dismutase, plasma ascorbic acid following 30 days of wheatgrass administration. It should be noted however, that both these studies used different sample populations, study designs, and biochemical parameters of oxidative stress, compared to the present study. Considering the nutraceutical product as used in the present study is a combined barley-wheat grass blend, the current data suggests that future research should consider a minimum of 14 days supplementation and/or a higher supplementation dose. It is important to highlight, that although outside the control of the researcher, it would be obtuse not to consider the horticultural limitations presented when investigating such species of grass. For one, there is evidence to suggest agricultural variables such as genotype, age, year, soil conditions, and locality all affect antioxidant concentrations within young barley and wheatgrass (Acar *et al.*, 2001; Ehrenbergerova *et al.*, 2009). Furthermore, Bamforth (1983) observed differences in superoxide dismutase activity when factors such as soil pH, location of growth, and processing variables (extraction, mashing, heat, malting etc) were modified during the growth of young barley shoots. The author is mindful that similar observations and the subsequent differences to the nutritional profile could be applied across multiple, if not all, food produce.

Due to RONS scavenging capabilities, an activated reduction in the concentration of LSA may coincide with a reduction in the biomarkers associated with an oxidative insult (Fogarty *et al.*, 2012). Both Traber & Atkinson (2000) and McAnulty *et al.* (2005), suggest that α-tocopherol concentration may be compromised within the vascular

circulation as a function of acute exercise. Contrary to this, antioxidant supplementation can increase systemic lipid- and aqueous-soluble antioxidant concentration (Rokitzki *et al.*, 1994; Subudhi & Mattson, 2000). Although not significant, α-tocopherol, γ-tocopherol, retinol, and xanthophyll increased following exhaustive exercise, perhaps suggesting an activation of lipolysis, and a subsequent antioxidant releasing effect into the circulation (Davison *et al.*, 2002). In agreement, both Long *et al.* (2008) and Fogarty *et al.* (2012), suggest this can be exacerbated when exercising in a fasted state. Supplementation of BWJ increased xanthophyll concentration only (Δ69.7%), and work has shown that xanthophyll is an effective antioxidant with regards protecting against DNA damage and lipid peroxidation (Haegele *et al.*, 2000). Although participants were under clear instruction to follow their habitual dietary intake, it is important to note that dietary intake was not strictly controlled *per se*, and it is indeed conceivable that a heterogeneous diet may influence the systemic concentration of LSA (Mayne, 2003; Eastep & Chen, 2015). However, within the present study, there was no difference observed in blood lipid soluble antioxidant concentrations between groups at the pre-exercise time point; thus, supporting the notion that diet remained constant across the study. Additionally, the authors are confident that the randomized, crossover design of the study minimised any significant dietary variation.

Recent research demonstrates an increase in SOD, GPx and glutathione reductase activity following exhaustive exercise to volitional fatigue (Bouzid *et al.*, 2014). Additionally, Khassaf and colleagues (2001), has shown an elevation in muscle cell SOD activity following a single bout of exhaustive exercise. The findings of the present study showed no difference between intervention groups or across time points. Nevertheless, and whilst taking into consideration the pooled (pre- vs. post-exercise) time points, the present data illustrates that supplementing with BWJ provides a Δ46% increase in SOD activity compared to a Δ30% increase in the placebo group. Considering the marginal reduction in DNA and lipid hydroperoxides damage following supplementation, alongside the elevation in extracellular SOD observed in the supplemented groups, it is theoretically plausible that BWJ provided a degree of cell protection against an exercise-induced oxidative insult; however, further research is warranted to confirm this supposition.

It should be noted that there are limitations with the current study, and any future experiment using this novel based nutraceutical should include more than double the existing sample size; assuming that any similar investigative study will use apparently

healthy male volunteers of a similar age and body mass index as that used in the current study. Although a prospective calculation of power was performed, calculating the power of the test retrospectively (power = 0.3) highlights the inherent weakness of performing such calculations prior to testing.

4.5 Conclusions

This investigation confirms previous work (from the laboratory of Davison, and others) that exhaustive exercise can cause DNA and lipid damage, and that the novel BWJ nutraceutical product used does not statistically attenuate the observed cell damage. That said, these findings may be viewed as preliminary evidence that this plant-derived nutraceutical supplementation may have some potential beneficial effects with regards reducing an exercise-associated oxidative insult, and further work in this area should focus on a revised participant number and the length of supplementation period. Moreover, it is imperative to determine the exact mechanistic based properties; it is therefore necessary to perform UHPLC-MS on the specific plant-derived nutraceutical to provide a better understanding of the constituents within the specific product.

Chapter Five

The DNA Damage-Repair Response Following High-Intensity Exercise in Hypoxia

5.0 Abstract

Introduction: This study examines the interplay between exercise and hypoxia in relation to the DNA damage-repair response; with specific interest to DNA double strand damage.

Methods: Following two $\dot{V}O_{2max}$ tests, 14 healthy, male participants completed two exercise trials (hypoxia; 12% FiO_2, normoxia; 20.9% FiO_2) consisting of cycling for 30-minutes at 80-85% of $\dot{V}O_{2max}$ relative to the environmental condition. Blood was sampled pre-, immediately post-, 2-, and 4-hours post-exercise with additional blood cultured *in vitro* for 24-, 48-, and 72-hours following the experimental trial. Samples were analysed for single and double strand DNA damage, base oxidation, lipid hydroperoxides, lipid soluble antioxidants, and the ascorbyl free radical.

Results: Exercise caused an increase in single strand and base oxidation as a result of exercise ($P < 0.05$) which was exacerbated following hypoxia ($P = 0.02$); similar increases in DNA double strand breaks occurred as a result of hypoxia ($P < 0.000$). With respect to the DNA damage-repair response, single strand, base oxidation, and double strand lesions were fully repaired by the 4-, 24-, and 48-hour time points respectively. Changes in lipid hydroperoxides ($P = 0.001$), the ascorbyl free radical ($P = 0.02$), and lipid soluble antioxidants ($P > 0.05$), were also observed following exercise in hypoxia.

Conclusions: These findings highlight significant single- and double strand DNA damage and oxidative stress as a function of high-intensity exercise, which is substantially exacerbated in hypoxia which may be attributed to multiple mechanisms of RONS generation. In addition, full repair of DNA damage (SSB, DSB, and base oxidation) was observed within 24- and 48-hours of normoxic and hypoxic exercise, respectively.

5.1 Introduction

Consensus within the literature states that regular exercise produces many biological and metabolic adaptations, which consequently, have been shown to reduce the susceptibility of several pathological ailments such as cardiovascular disease, neurodegenerative disease, and cancer (Booth *et al.*, 2017). Despite this, the literature is clear that prolonged and/or strenuous exercise, produces detrimental pathophysiological effects through the significant generation of RONS (Alfadda & Sallam, 2012). In turn, this can result in a wide range of biological alterations impairing important cellular processes including signal transduction and transcription, and the alterations and downstream consequences to deoxyribonucleic acid (DNA) integrity (Liu *et al.*, 2018).

It appears that both acute high-intensity, and prolonged endurance exercise, causes a substantial increase in RONS generation, and consequential modifications to DNA; indicating an intensity and/or duration threshold by which exercise is sufficiently challenging to overcome the natural antioxidant line of defence (Finaud *et al.*, 2006; Kawamura & Muraoka, 2018). Work from the laboratory of Davison and colleagues has demonstrated considerable damage to DNA strands following exhaustive running (Fogarty *et al.*, 2011), with others showing similar data from various exercise modalities (Yasuda *et al.*, 2015, Soares *et al.*, 2015). Data presented in chapter 4 of this thesis (Williamson *et al.*, 2018) supports published research providing additional evidence for DNA single strand breaks following exhaustive exercise. To the best of the authors knowledge, Lippi *et al.* (2016) was the first study to display DNA double strand breaks (DSBs) following aerobic endurance exercise from 5- to 42-km as expressed by detection of *γ*-H2AX foci. Furthermore, there was a positive association between the incremental increase in exercise duration/intensity, and DNA injury. More recently, the same research group confirmed this increase in DSBs, following a 21-km endurance trial in both healthy and diabetic amateur runners (Lippi *et al.*, 2018).

With specific interest to double-stranded DNA damage, a repair response is initiated at the onset of strand damage through the activation of the serine/threonine protein kinases ataxia-telangiectasia-mutated (ATM), ataxia-telangiectasia-rad3-related (ATR), DNA-dependant protein kinase, and subsequent phosphorylation of SER139 of the histone H2AX (Lassmann *et al.*, 2010); commonly termed, *γ*-H2AX. It should be noted, the sole use of *γ*-H2AX foci to quantify DSBs is polemical, with

evidence now suggesting that *γ*-H2AX is associated with other types of cellular stressors such as ionizing radiation (Stiff *et al.*, 2004), bleomycin (Banath & Olive, 2003), pH (Xiao *et al.*, 2003), mono-functional alkylating agents (Stojic *et al.*, 2004), replication stresses (Liu *et al.*, 2003), meiotic recombination (Mahadevaiah *et al.*, 2001), apoptotic DNA fragmentation (Rogakou *et al.*, 2000), senescence (Sedelnikova *et al.*, 2004), dysfunctional telomeres (Hao *et al.*, 2004) and heat stress (Takahashi *et al.*, 2004; Kaneko *et al.*, 2005;). Furthermore, there is evidence demonstrating that *γ*-H2AX participates in both the non-homologous end joining, and homologous recombination repair pathways of DNA DSBs (Paull *et al.*, 2000; Chronis & Rogakou, 2007); thus, when investigating the DNA damage-repair response, exclusive use of *γ*-H2AX will provide limited mechanistic data. As such, the analysis and use of co-localised repair proteins such as 53-Binding Protein 1 (53BP1), RAD51, BRCA1 and NBS1, to further optimise the sensitivity of quantifying DNA double-stranded damage should be considered (Ward *et al.*, 2003). Specifically, there is a positive correlation between the *γ*-H2AX-53BP1 co-localisation at the site of double strand DNA damage; thus, co-immunostaining for *γ*-H2AX and 53BP1 is a widely accepted marker indicating the presence of a double strand break (Nikolova *et al.*, 2014).

Although acute and chronic hypoxic exposure modifies redox balance (Strapazzon *et al.*, 2016), the addition of exercise in a hypoxic environment further exacerbates oxidative damage. In a seminal study, Møller and colleagues (2001), observed an increase in DNA strand breaks and oxidative damage following hypoxic exercise at altitude (4559m above sea level). It was inferred that the observed DNA damage was a result of impairments in the antioxidant capacity and an activation of poly(ADP)-ribose polymerase despite not quantifying these markers within their study.

At present, no study has comprehensively quantified the effect of hypoxia and exercise on both single- and double-stranded DNA damage, using co-immunofluorescence of *γ*-H2AX and 53BP1. This study will aim to provide a robust understanding of the lymphocyte cell DNA damage-repair response and explore the interplay of systemic measures of oxidative stress (such as lipid peroxidation, and lipid soluble antioxidants) within this relationship. The evidence surrounding the DNA-damage repair response as a function of high-intensity exercise is limited; this study seeks to investigate if any residual DNA damage is detectable for up to 72 hours following the oxidative insult.

5.2. Materials and Methods

5.2.1. Participants

Fourteen (*n*=14) apparently healthy, recreationally active males (age 22 ± 2 years, height 178 ± 6 cm, weight 83 ± 8 kg), volunteered for the study and provided their medical history, along with written informed consent, prior to commencing the study. All participants were non-smokers and free from any form of medication or antioxidant supplementation for 4 weeks prior, and throughout, the study. Any participants who were recently exposed to altitudes higher than 1500m were excluded from the study. The study was conducted in accordance with the Declaration of Helsinki and approved by a local University Ethics Committee (REC/16/0004; See Appendix A).

5.2.2. Preliminary Preparation

Prior to experimental testing, all participants completed two conditional $\dot{V}O_{2max}$ tests (normoxic and hypoxic) to volitional fatigue with no longer than 7-days between tests. Physiological outcomes are displayed in Table 5.1.

Table 5.1. Experimental physiological variables.

	Normoxia	**Hypoxia**
HR_{rest} ($b.min^{-1}$)	67 ± 8	76 ± 9*
HR_{max} ($b.min^{-1}$)	190 ± 9	175 ± 12*
$\dot{V}O_{2max}$ ($mL{\cdot}kg^{-1}{\cdot}min^{-1}$)	53.2 ± 10	42.8 ± 8*
SaO_2 (%)	97 ± 0.7	77 ± 4*
TTE (mins)	13 ± 2.8	12 ± 2.1

*HR = Heart rate; TTE = Time to exhaustion. * P < 0.05 compared with normoxia.*

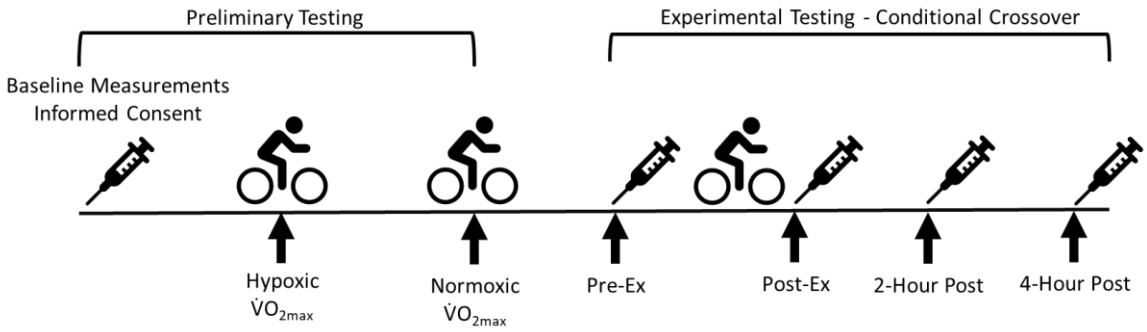
5.2.3. Research Design

Participants abstained from exercise and alcohol for 48 hours before completing all preliminary and experimental testing. All participants were fasted for 12-hours before experimental testing; this approach was necessary to standardize inter-participant blood biochemistry. To negate the possibility of diurnal variation, all participants attended the laboratory at the same time of day (08:00) for each experimental phase. All experimental trials were performed in the same normobaric hypoxic chamber set at 20.9% FiO_2 and 12% FiO_2 (≈ 4600m) for normoxic and hypoxic conditions respectively; humidity and temperature were set to 50%, and 16°C for all trials. Participants cycled for 30-minutes at 80-85% of relative $\dot{V}O_{2max}$. Participants could drink water *ad libitum*.

5.2.4. Biochemical Analysis

Blood was extracted from a prominent antecubital forearm vein pre- and immediately post-exercise; in addition, blood samples were obtained 2-hours and 4-hours post-exercise. All blood was extracted using an intravenous cannula (Introcan Safety 3, Braun, Germany), and subsequently centrifuged, aliquoted, and stored at -80°C prior to biochemical analysis. A schematic overview of the study and blood draws is shown below in Figure 5.1.

Figure 5.1. Schematic overview of experimental protocol.



Participants completed a $\dot{V}O_{2max}$ test in both conditions before commencing the experimental trials in either normoxia and hypoxia.

An exercise-induced haemoconcentration was determined using the equations of Dill and Costill (1974), incorporating the haemoglobin and haematocrit indices; this was used to account for acute-exercise induced plasma volume changes. Packed cell volume (%) was measured using the microcapillary reader technique and corrected by 1.5% for plasma trapped within erythrocytes (Dacie and Lewis, 1968). It should be noted, all DNA analysis, as outlined in Section 5.2.5., was performed on the same aliquot to reduce variation between assays.

5.2.5. DNA Single Strand Breaks and Base Oxidation

DNA damage was measured in human peripheral blood mononuclear cells (PBMCs) using the single cell gel electrophoresis, or comet assay (Davison, 2016) as per section 3.4.11.. The intra/inter-assay coefficients of variation (CV's) of this assay is <8%.

5.2.6 Detection of Double Strand Breaks via γ-H2AX and 53BP1

DNA double strand breaks were measured using dual-staining immunohistochemistry as fully described in section 3.4.5.2.

5.2.7. Lipid Hydroperoxides (LOOH)

Serum LOOH was measured spectrophotometrically using the method of Wolff (1994) as outlined in section 3.4.3. The intra/inter-assay coefficients of variation (CV's) of this assay is <5%.

5.2.8. Lipid Soluble Antioxidants (LSA)

LSA were analysed by simultaneous determination using the high-performance liquid chromatography (HPLC) method as per section 3.4.4. The intra/inter-assay coefficients of variation (CV's) of this assay is <7%.

5.2.9. Electron Paramagnetic Resonance (EPR)

The ascorbyl free radical was measured using EPR on a Bruker EMX EPR spectrometer (Bruker Instruments Inc., Billerica, MA, USA). A full protocol is in section 3.4.2.1.

5.2.10. Cell Culture

Additional primary lymphocytes were isolated from participant's blood samples immediately post-exercise for determining DNA repair at 24hr, 48hr and 72hr time points. Cells were cultured and prepared for analysis at the time points of interest as described in section 3.5.

5.2.11. DNA Damage Positive Control

Resting human lymphocytes were used as a positive control prior to comet assay analysis. 50 µL of cells were incubated with increasing concentrations of hydrogen peroxide for 30 minutes at 4°C to prevent DNA repair.

5.2.12. Statistical Analysis

The method of Cohen was utilized to calculate the power of the test based on DNA damage data published by Fogarty *et al.* (2011). SPSS statistical software (IBM, Surrey, UK, v.25) was used to analyse data sets, and data normality was determined using the Shapiro-Wilks test ($P > 0.05$). A two-way, repeated-measures ANOVA ascertained differences between groups and across time. Following a significant interaction effect, between group differences were analysed using a one-way ANOVA,

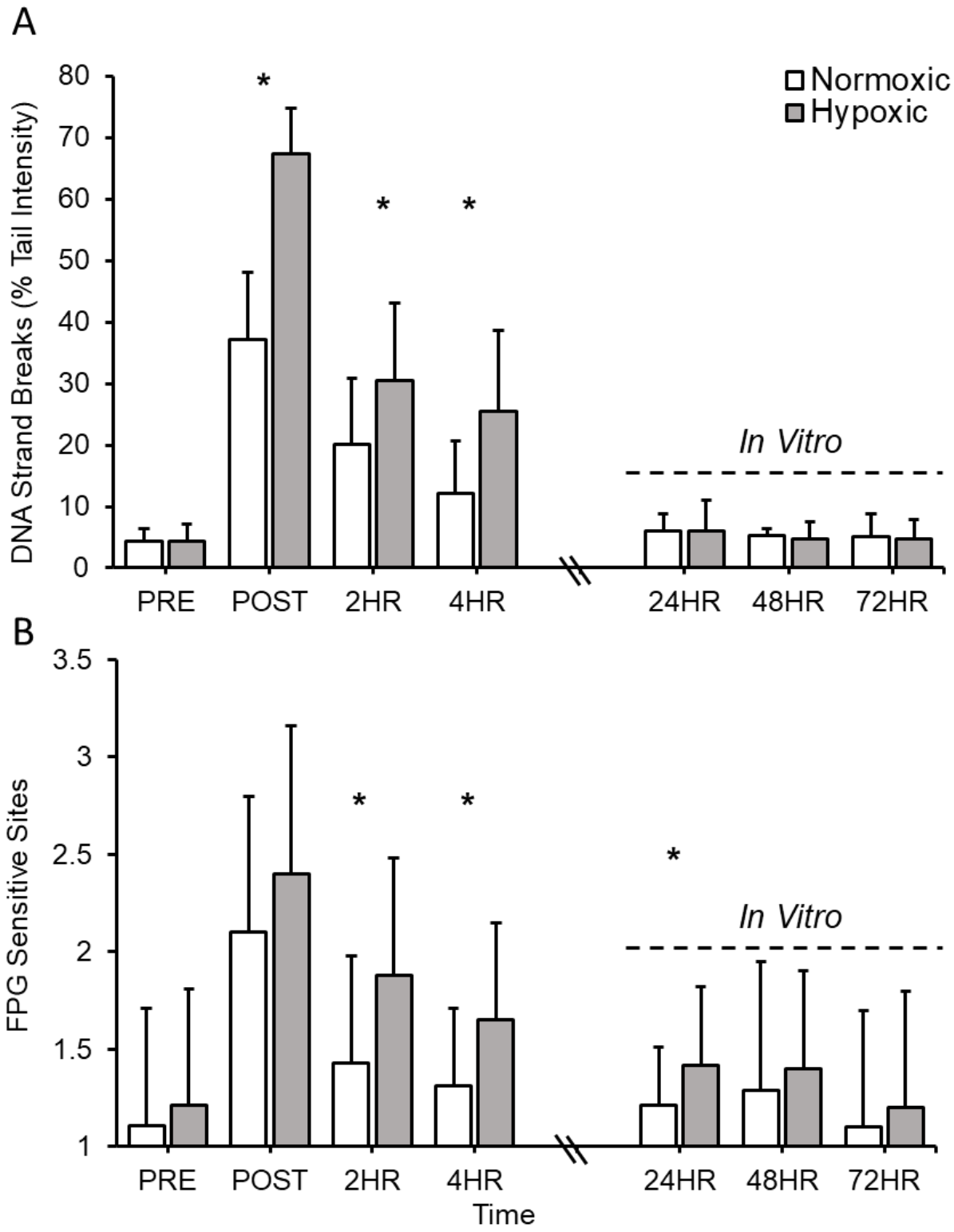
while a Bonferroni paired samples t-test was used for the within time differences. All significant changes were established at 95% confidence intervals ($P < 0.05$). All data expressed as mean ± standard deviation unless otherwise stated. The magnitude of change was expressed as partial eta squared (effect size, ES) throughout.

5.3 Results

5.3.1. DNA Damage and Base Oxidation

The time-course of DNA single-stranded breaks (expressed by % tail intensity) following high-intensity exercise, as depicted in Figure 5.2A, demonstrates an interaction effect of time x group ($P < 0.05$, ES = 0.44). There was a significant increase in DNA single strand damage from pre- to post-exercise; returning to baseline by the 24-hour time point. Post-hoc analysis indicates a significant difference between the normoxic and hypoxic conditions immediately post- ($P = 0.000$, ES, 0.7), 2-hour post- ($P = 0.03$, ES = 0.17) and 4-hour post-exercise ($P = 0.004$, ES = 0.28). Figure 5.2B depicts the level of FPG-sensitive sites as a result of high-intensity normoxic and hypoxic exercise. Base oxidation increased as a function of exercise (pooled data) from the pre- to post-exercise time point. There was an interaction effect of time x group ($P = 0.02$, ES = 0.11), and the post-hoc analysis identified a difference at the 2-hour post- ($P = 0.001$, ES = 0.36), 4-hour post- ($P = 0.01$, ES = 0.21), and 24-hour post-exercise ($P = 0.03$, ES = 0.17) time points. Collectively, this data illustrates an increase in DNA damage and base oxidation for up to 4-hours following high-intensity hypoxic exercise, with FPG-sensitive sites being detected for up to 24-hours following insult.

Figure 5.2. Time-course of DNA damage and base oxidation as expressed by the comet assay and FPG-incubation respectively within the conditional experimental trials.

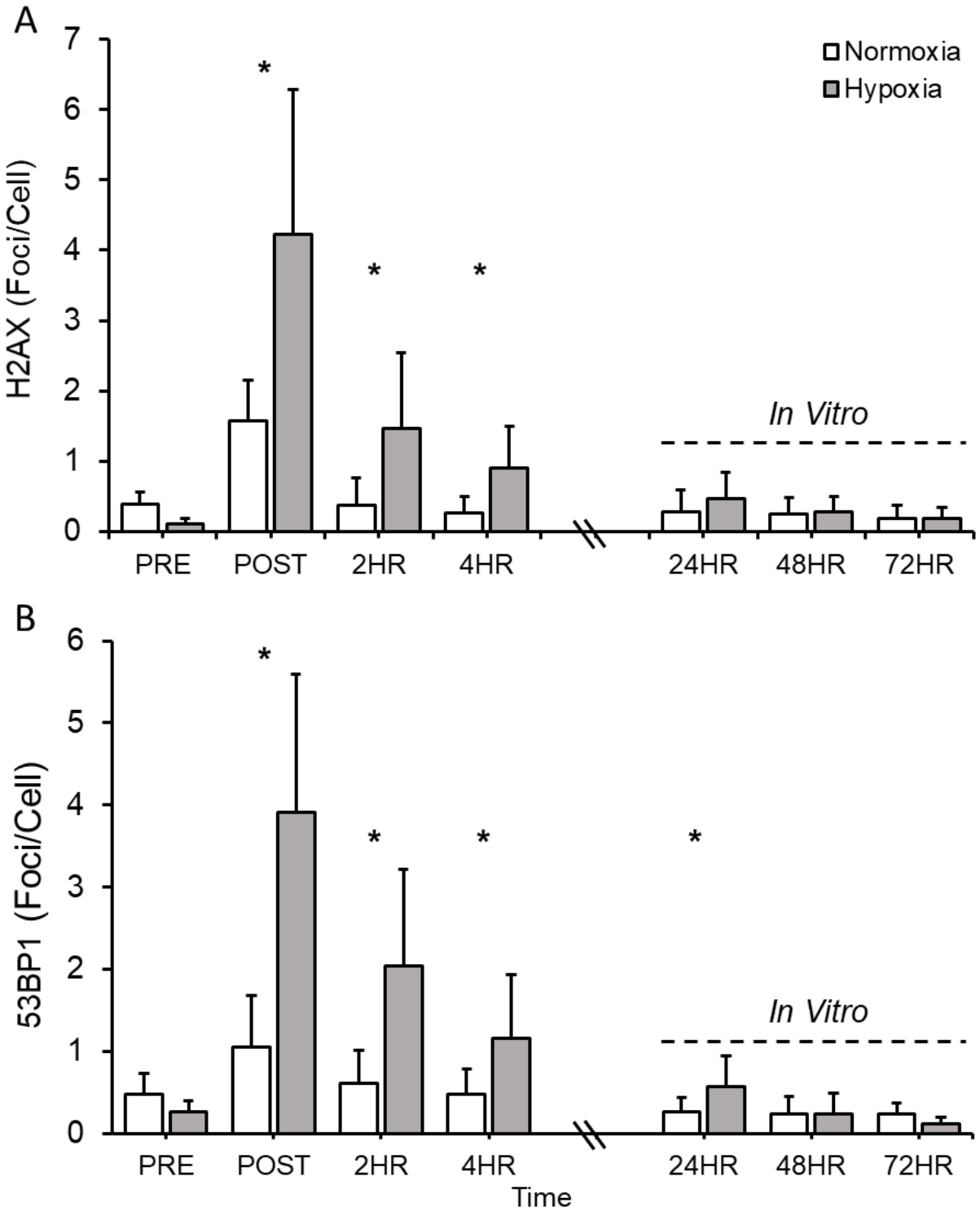


** P < 0.05 represents the significant interaction effect of time x group. All data expressed as mean ± standard deviation.*

5.3.2. H2AX Phosphorylation and 53BP1

The time-course of histone phosphorylation (expressed as average foci per cell) in peripheral blood lymphocytes following exercise in normoxic and hypoxic conditions is illustrated in Figure 5.3. There was an increase in y-H2AX detection from 0.39 ± 0.18 foci per cell pre-exercise to 1.57 ± 0.59 foci per cell post-exercise in the normoxic group ($P < 0.000$). This would indicate that high-intensity normoxic exercise is sufficiently challenging to cause DNA DSBs. However, there was also a reduction ($P < 0.000$) in the amount of phosphorylated H2AX foci per cell from immediately post-exercise to 2-hours post-exercise (1.19 foci per cell), indicating a rapid DNA damage-repair response. With regards to experimental conditions, there was a significant interaction effect of time x group ($P < 0.000$, ES = 0.67) with post-hoc analysis indicating an increase from pre-exercise to immediately post- ($P < 0.000$, ES = 0.45), 2-hour post- ($P = 0.001$, ES = 0.33), and 4-hour post-exercise ($P = 0.001$, ES = 0.34) time points in the hypoxic group. Analysis of 53BP1 foci shows similar trends over the time course with a significant effect of time x group ($P < 0.000$, ES = 0.53) as a function of exercise. Additionally, post-hoc analysis indicated an increase in 53BP1 detection within the hypoxic group from pre-exercise to immediately post- ($P < 0.000$, ES = 0.57), 2-hour post- ($P < 0.000$, ES = 0.42), 4-hour post-exercise ($P = 0.005$, ES = 0.27), and 24-hour post-exercise ($P = 0.009$, ES = 0.23) time points. Cumulatively, these results indicate that high-intensity exercise causes significant double stranded DNA damage as expressed by H2AX and 53BP1 detection, with this oxidative insult being exacerbated in hypoxic conditions.

Figure 5.3. Time-course of γ-H2AX and 53BP1 yield within the conditional experimental trials.

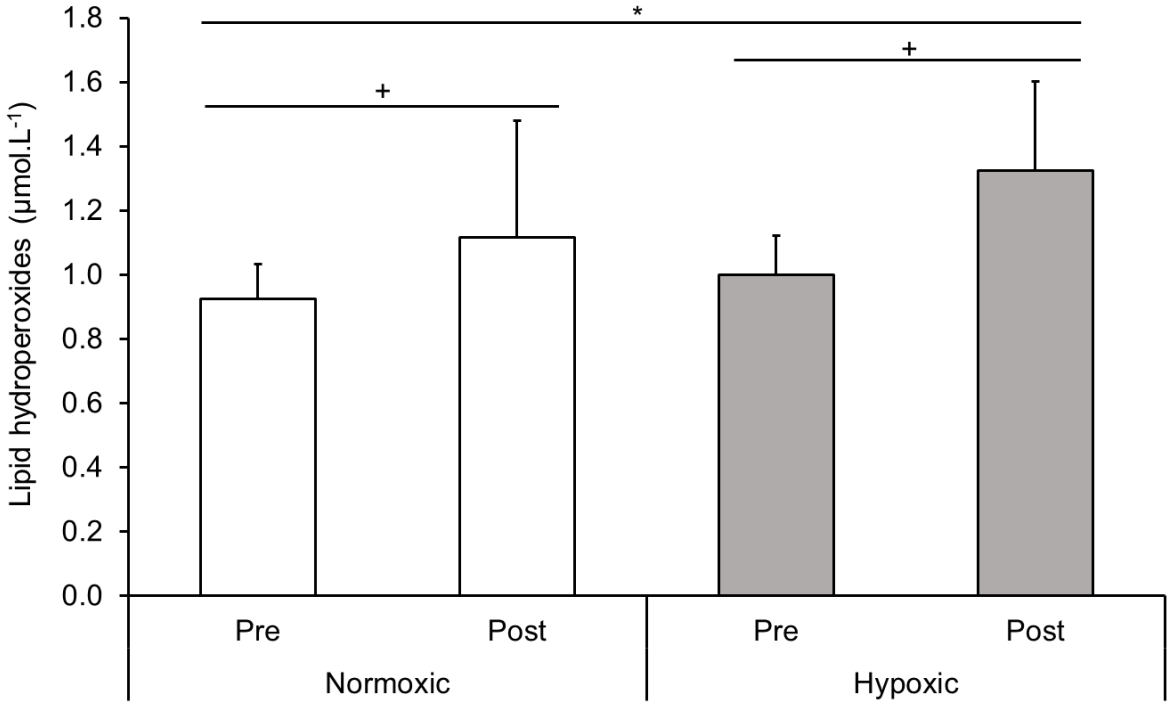


**P < 0.05 represents the significant interaction effect of time x group. All data expressed as mean ± standard deviation.*

5.3.3 Lipid Hydroperoxides

There was a significant effect of time x group (P = 0.03, ES = 0.2) as observed in Figure 5.4. Lipid hydroperoxides were elevated (P = 0.001, ES = 0.3) post-exercise in the hypoxic group in comparison to the normoxic condition. Furthermore, there was also a significant effect of time at the post-exercise time point in both conditions. Within normoxic conditions, LOOH increased significantly (P < 0.000) by 0.14 µmol·L^{-1} as a function of exercise, with the hypoxic group increasing by 0.33 µmol·L^{-1} (95% CI, 0.15 to 0.50) (P = 0.002); indicating that high-intensity exercise is capable of causing oxidative insult to lipids, irrespective of group.

Figure 5.4. Conditional lipid hydroperoxides as a function of high-intensity exercise.



** denotes a significant interaction effect of time x group (P < 0.05). + represents a significant effect of time (P < 0.005). All data expressed as mean ± standard deviation.*

5.3.4. Lipid Soluble Antioxidants

There was no interaction effect of time x group ($P > 0.05$) for any of the antioxidants as represented in Table 5.2. Main effects of time ($P < 0.05$) were observed in *α*-carotene (ES = 0.54), lycopene (ES = 0.65), *γ*-tocopherol (ES = 0.43), and *α*-tocopherol (ES = 0.41).

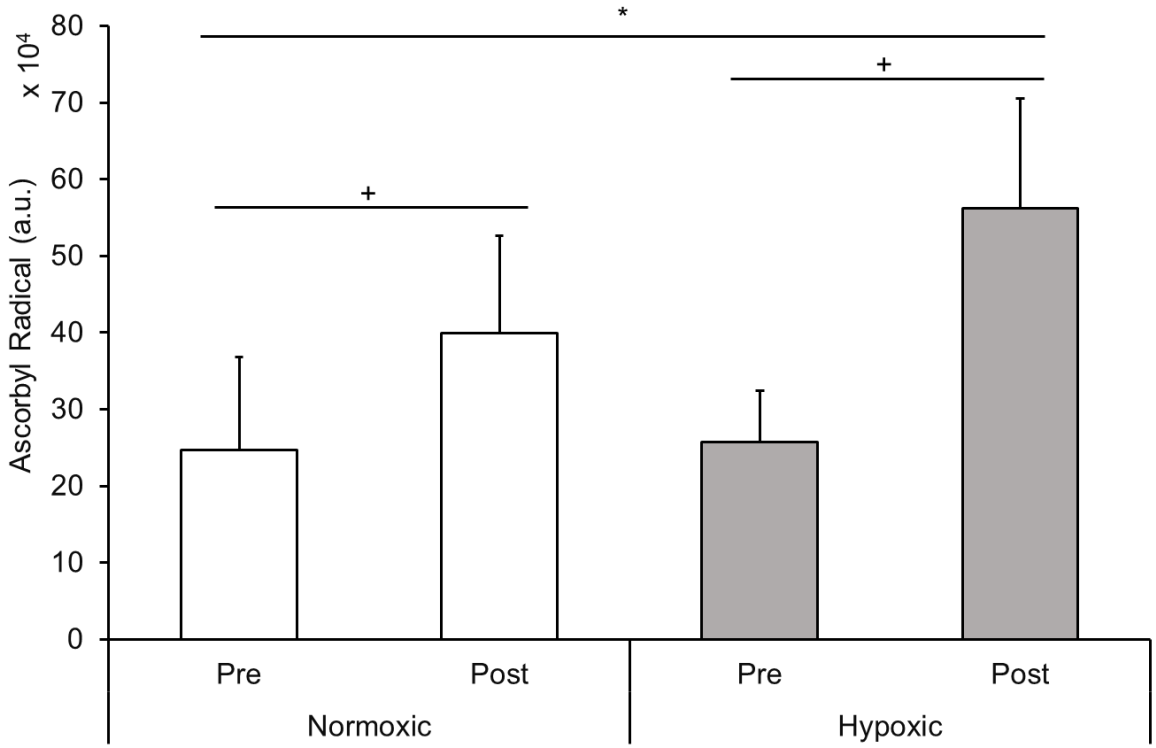
*Table 5.2 - Lipid soluble antioxidants at the pre-exercise and post-exercise time points for the conditional trials. All values are presented as means ± SD and expressed as mmol·L^{-1}. * denotes a main effect of time (pooled data; p < 0.05).*

	Normoxic			**Hypoxic**		
	Pre	Post	%Δ	Pre	Post	%Δ
γ-Tocopherol	1.81±0.9	1.44±0.6*	-20	1.93±0.6	1.46±0.4*	-24
α-Tocopherol	22.27±7.2	18.31±6.8*	-18	26.69±6.7	20.58±7.8*	-23
Retinol	2.42±0.6	2.35±0.6	-3	2.69±0.5	2.49±0.4	-7
Lycopene	0.27±0.1	0.17±0.04*	-37	0.32±0.1	0.16±0.1*	-50
α-Carotene	0.02±0.01	0.01±0.01*	-23	0.02±0.01	0.01±0.01*	-40
β-Carotene	0.15±0.1	0.13±0.1	-9	0.17±0.1	0.14±0.1	-18

5.3.5. Ascorbyl Radical

As observed in Figure 5.5, there was a significant interaction effect between time and group (P = 0.022, ES = 0.46). Additionally, there was a significant effect of time within each group (P < 0.05) as a consequent of high-intensity exercise. This data would indicate that high intensity exercise causes significant oxidation of ascorbic acid, thus producing the ascorbyl radical; furthermore, production of this oxidative stress biomarker is exacerbated as a function of exercise in a hypoxic environment.

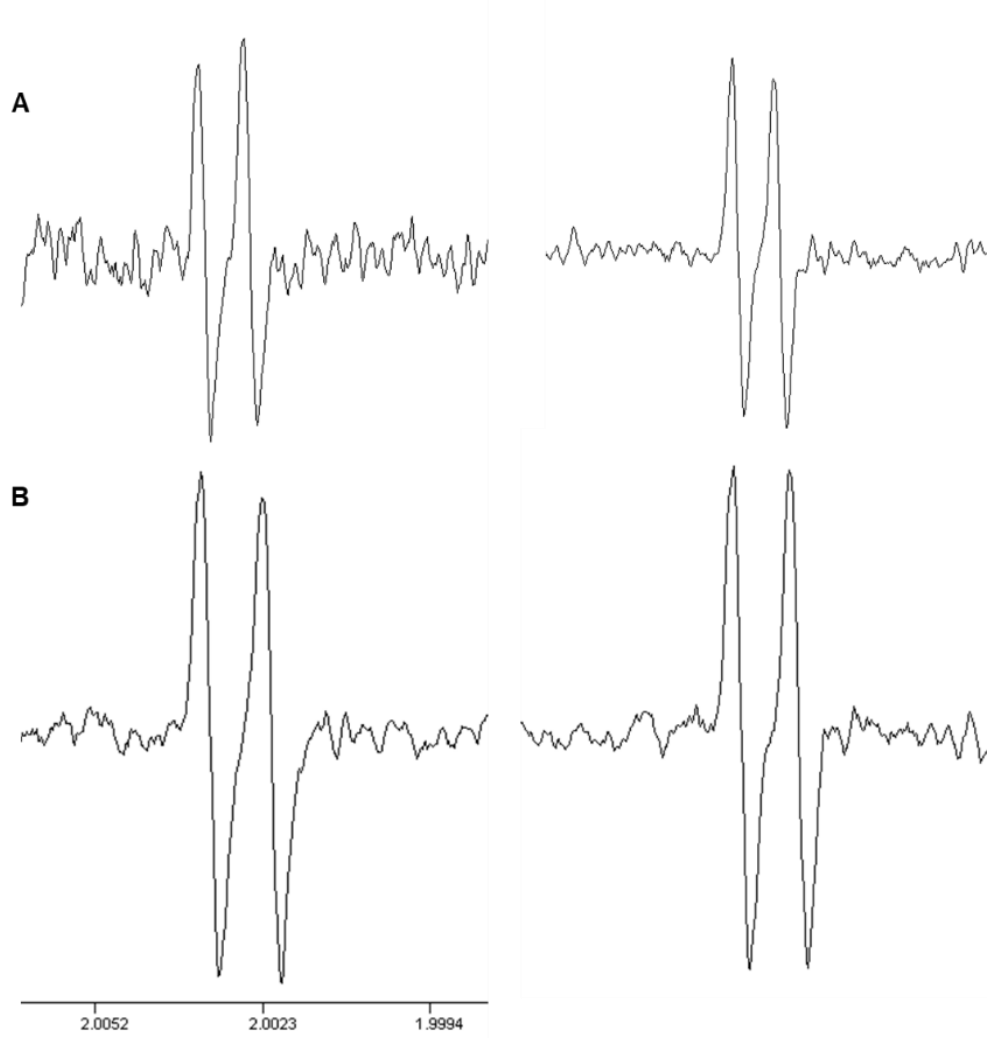
Figure 5.5. Ascorbyl free radical as a function of high-intensity exercise.



** denotes a significant interaction effect of time x group (P < 0.05). + represents a significant effect of time (P < 0.05). All data expressed as mean ± standard deviation. Abbreviations – a.u. = arbitrary unites.*

Ascorbyl free radical concentration was detected at the immediate post-exercise time point for the normoxic and hypoxic trials. Representative ascorbyl radical spectra pre and post exercise are displayed in Figure 5.6.

*Figure 5.6. Representative ascorbyl free radical spectra obtained at (**A**) pre-exercise, and (**B**) immediately post-exercise in 12% hypoxia.*

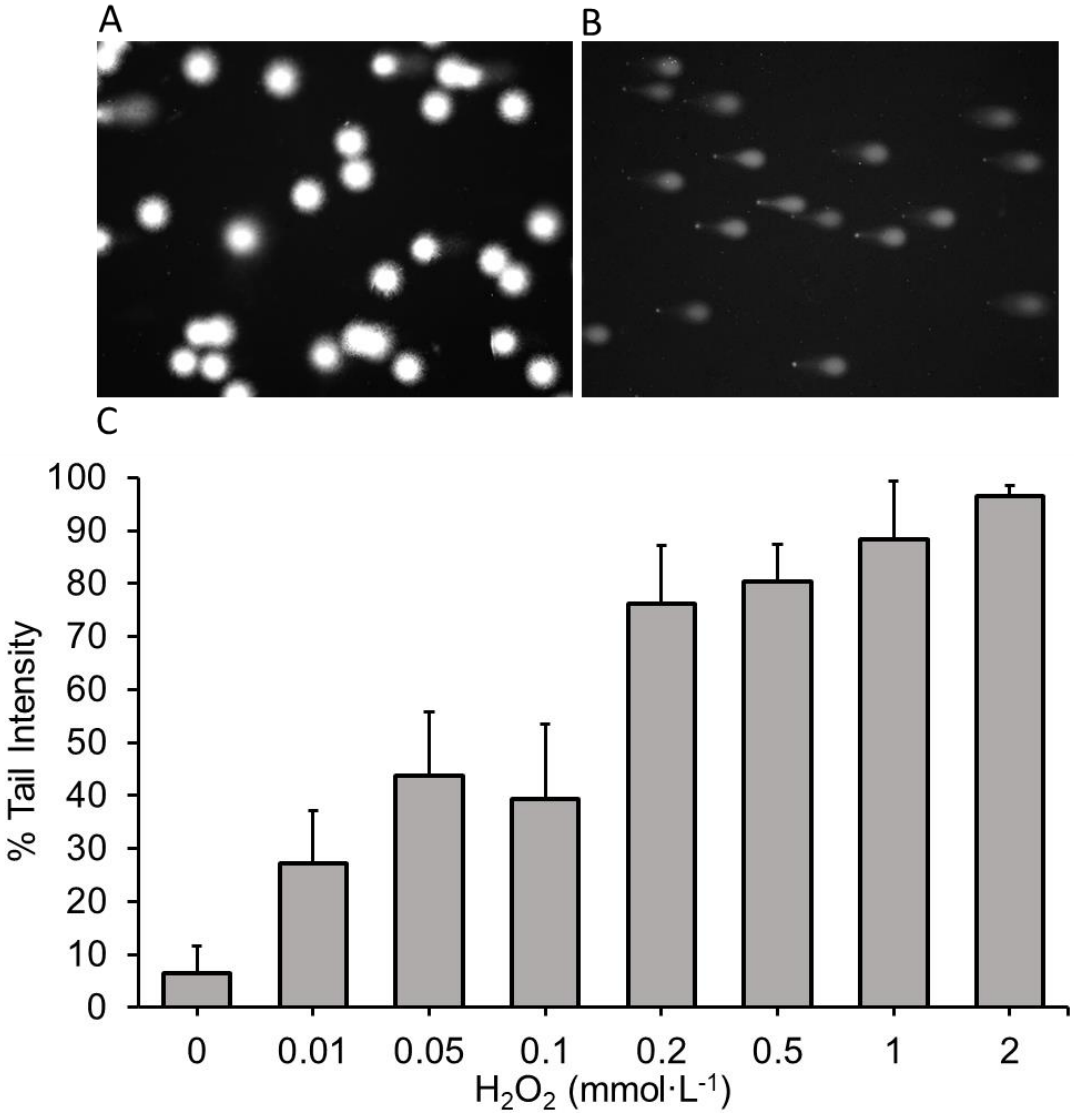


All spectra were filtered and scaled identically.

5.3.6. Hydrogen Peroxide Positive Control

DNA damage, as determined by the comet assay and expressed by tail intensity, demonstrated a dose-dependent relationship as hydrogen peroxide concentration increased as illustrated in Figure 5.7. The observed damage from 0 to 0.1 mmol·L^{-1} is similar to that expressed by the isolated lymphocytes following exercise-induced oxidative damage in Figure 5.2A. Concentrations of hydrogen peroxide beyond 0.1 mmol·L^{-1} induced substantially more damage.

Figure 5.7. Hydrogen peroxide series.



*Note; **A** Typical sample captured from 0 mmol·L^{-1} H_2O_2, **B** Typical sample captured from 0.2 mmol·L^{-1} H_2O_2, **C** Positive control DNA damage (expressed as % tail intensity) as assessed incubating lymphocytes in different concentrations of H_2O_2 for 20 minutes. All data expressed as mean ± standard deviation.*

5.4 Discussion

The aim of this study was to characterise the effects of high-intensity exercise on single- and double-stranded DNA damage exercise in hypoxia. Additionally, the DNA damage-repair response for up to 72-hours following the oxidative insult was ascertained. This study demonstrates significant DNA damage and oxidative stress as a function of high-intensity exercise, which is substantially exacerbated in hypoxia.

5.4.1. DNA Damage

The findings of this study confirm that strenuous exercise produces significant oxidative damage to DNA, and other biological macromolecules, possibly through the generation of RONS. This relationship was initially confirmed through the work of Hartmann *et al.* (1994) but has been replicated successfully over a multitude of exercise modalities (Fogarty *et al.*, 2011; Fogarty *et al.*, 2013). Although the narrative, and contribution, surrounding the mechanisms associated with RONS generation remains contentious, NADPH oxidase, the mitochondrial electron transport chain, and xanthine oxidase, are often cited as the predominant sources during exercise (Steinbacher & Eckl, 2015; He *et al.*, 2016). With regards to hypoxic exposure, the pioneering study by Møller and colleagues (2001), demonstrated substantial DNA strand breaks and oxidative base damage; supported by the present study, this oxidative insult was intensified following strenuous, hypoxic exercise.

The literature examining exercise-induced oxidative stress and DNA damage, predominantly focuses on single-strand breaks through the use of the comet assay (Azqueta *et al.*, 2014), with studies also including base-oxidation via enzyme incubation of ENDO III, FPG or hOGG1 (Soares *et al.*, 2015). Briviba *et al.* (2005) observed no change in single strand DNA damage or FPG sensitive sites but showed an increase in ENDO III sensitive sites as a function of competitive marathon and half-marathon running. In support of these findings, Reichhold *et al.* (2008) and Neubauer *et al.* (2008) found similar responses in single strand damage, FPG- and ENDO III sensitive sites as a result of Ironman triathlons. However, DSBs have greater biological significance and could contribute to cell apoptosis, and genomic instability; yet, the experimental evidence associated with exercise and DNA DSBs is lacking. Lippi and colleagues (2016) identified a dose-dependent response between exercise duration and *γ*-H2AX detection, with maximum damage occurring following a 42-km run. More recently, Lippi *et al.* (2018) demonstrated an increased detection of phosphorylated

H2AX following 21-km of running in healthy amateur runners; this response was exacerbated in a diabetic group. It was postulated that the synergistic combination of exercise-induced oxidative stress, and the impairments in glucose metabolism led to the excess production of RONS, thus compromising DNA stability.

Phosphorylated H2AX has been a hallmark of DNA DSBs in cancer and genotoxicity research for over a decade due to its sensitivity, specificity, and short analysis time (Sanchez-Flores *et al.*, 2015). Following induction of a DSB, *γ*-H2AX is rapidly recruited to the site of damage via one or more of the phosphatidylinositol 3-OH-kinase-like family; typically, by the MRE11-RAD50-NBS1 complex (Falck *et al.*, 2005; Lee & Paull, 2005). The correlation between the number of *γ*-H2AX foci, and the exact number of DSBs, remains controversial (Lobrich *et al.*, 2010); however, it is well accepted that *γ*-H2AX detection characterises a DNA DSB, while disappearance of *γ*-H2AX foci characterises repair of said damage (Mariotti *et al.*, 2013). Although *γ*-H2AX is considered a reliable indicator of DNA DSBs, many cancer chemo- and radio-therapy trials use colocalization of other DSB repair and recognition proteins such as 53BP1 and RAD51 to provide further insights of the DNA damage and repair pathways. It is understood that these recruitment and signalling factors of DSBs, initially act independently to H2AX, before subsequently being assembled at the site of damage in a colocalized manner (Celeste *et al.*, 2003).

The results of the present work demonstrate an increase in *γ*-H2AX and 53BP1 foci detection as a function of high-intensity exercise; with markers exaggerated in hypoxia. Although there is a clear increase in foci detection within normoxia, the challenge of hypoxia created a 2.5- and 3.5-fold increase in *γ*-H2AX and 53BP1 foci respectively following exercise. The elevations in *γ*-H2AX observed in the present study, compares with the data presented by Lippi *et al.* (2016) following 21-km of running in trained adults, and Djuzenova *et al.* (2015) following 0.5 Gy of X-ray exposure to human lymphocytes for 30 minutes. The mechanisms of DNA damage, including DSBs, have been comprehensively covered in the literature (Chatterjee & Walker, 2017), and described in section 2.7.1 of this thesis. However, these mechanisms differ slightly in hypoxia, and may explain the observed variation between conditions. During hypoxia, it is highly probable that mitochondria become inefficient, and shift to a more reductive state; thus, causing the spontaneous and/or enzyme-catalysed generation of superoxide (sometimes referred to as a superoxide burst; Hernansanz-Agustin *et al.*, (2014), perhaps due to a high proton motive force,

increased ubiquinol/ubiquinone, and increased NADH/NAD^+ (Loscalzo, 2016). It is also plausible that hypoxia increases vascular epinephrine, which is maximally stimulated during the initial exposure to acute hypoxia as arterial saturation declines (Mazzeo *et al.*, 1998). It also cannot be excluded that this physiological response to acute hypoxia may activate auto-oxidation of catecholamines, consequently increasing the generation of damaging RONS. Finally, Yuan *et al.* (2003) demonstrates that hypoxia-induced activation of the xanthine oxidase pathway can also produce RONS.

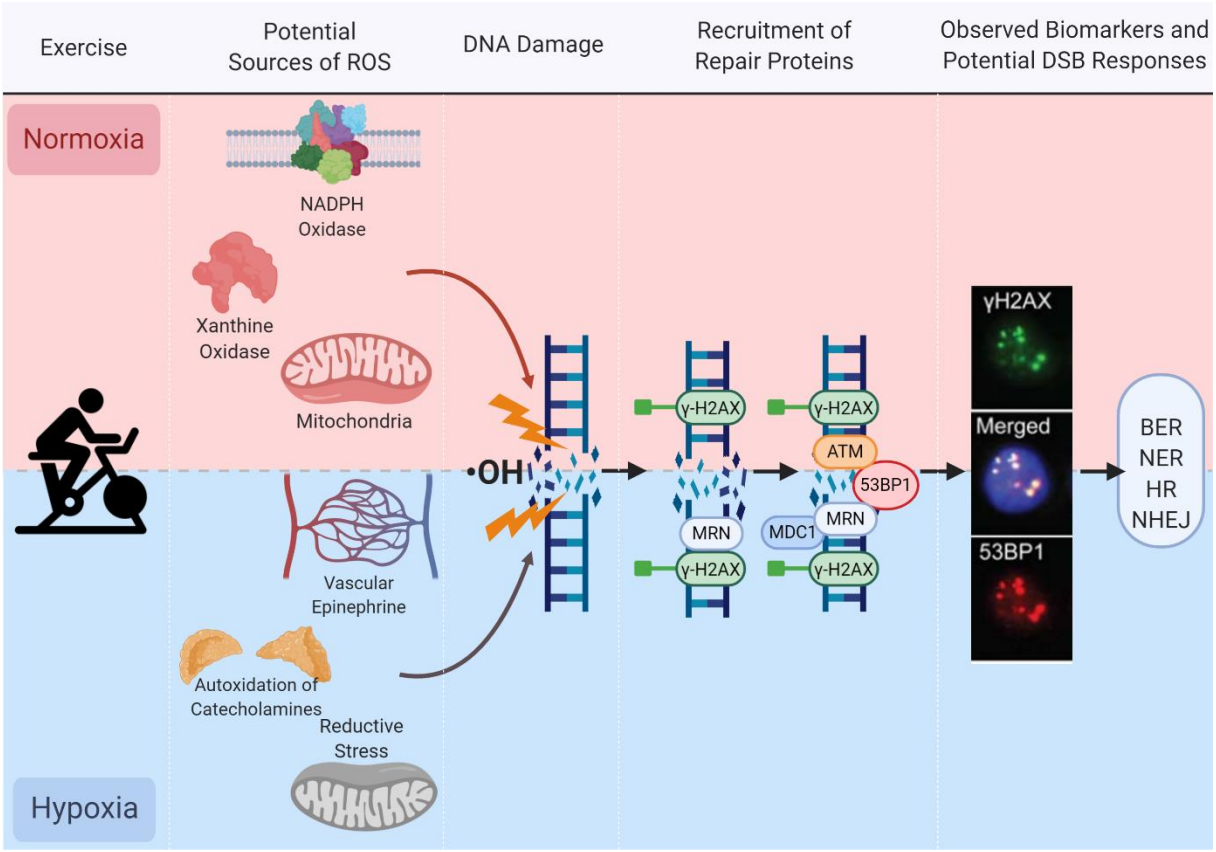
5.4.2 DNA Repair Response

The present data also characterises the DNA damage-repair kinetics following exercise. According to Collins & Squires (1986), the rate of DNA repair is determined by the intensity and severity of the DNA damage. Upon further inspection of results, DNA repair of single-stranded damage and FPG sensitive sites was achieved by the 24-hour time point within the normoxic group. A similar response was observed in the hypoxic group except for base oxidation as assessed by FPG-incubation, which required 48-hours to return to baseline. However, this author postulates that DNA repair was completed sooner than the 24-hour mark; future research should include an additional 6 - 8-hour post-exercise time point to further elucidate the repair pathway timeline. In support of this claim, following administration of x-rays (1 Gy), lymphocyte DNA damage as assessed by single-cell gel electrophoresis assay is fully repaired within 70-minutes (Cebulska-Wasilewska, 2003). Additionally, Palyvoda *et al.* (2003), concluded that full repair of human lymphocyte DNA single strand damage (as assessed by the comet assay) is achieved at approximately the 5-hour time point following 2 Gy of irradiation. With regards to base oxidation, it is likely that residual damage is being detected at the 24-hour time point. Incorporating FPG-incubation allows for greater sensitivity to changes in DNA damage, and can detect lower levels of DNA damage, while also identifying various DNA lesions such as 8-oxodG cleavage, FaPyGua, FaPyAde and other ring-opened purines (Miklos *et al.*, 2009). Commonly, oxidative DNA damage is repaired through the base excision repair (BER) pathway (Cadet & Davies, 2017). Specifically, oxidative damage is recognised and nicked by specific glycosylase and apurinic endonucleases (Van Houten *et al.*, 2018). Downstream repair proteins are recruited, specifically polymerase, XRCC1 and PARP1, to fill the apurinic site and subsequently ligated by DNA ligase III (Copeland & Longley, 2008; Lee & Wallace, 2017).

In relation to DNA DSB repair, *γ*-H2AX and 53BP1 demonstrated full repair within 2 and 4-hours respectively following exercise in normoxia. Although there was a clear increase, and subsequent decrease, in these biomarkers, it is important to note, the relative foci counts are still below the threshold for which would be considered deleterious. Typically, in genomic and biodosimetry research, baseline control cells would be considered viable with <2 foci per cell, while cells with large amounts of DSBs could reach 30-50 foci per cell (Ivashkevich *et al.*, 2012). While normoxia demonstrated rapid DNA repair, DNA repair in hypoxia was slightly slower, with *γ*-H2AX and 53BP1 foci fully repaired within 4 and 24-hours respectively. Following large doses of radiation (4 Gy), 75% of DNA damage is normally repaired within 7-hours post-exposure, while only residual foci remain at the 24-hour point (Sharma *et al.*, 2015); even when cells were exposed to 2 Gy of 225 kV_p X-rays split into 2 exposures of 1 Gy, data suggests full repair happens within 24-hours (<5 foci/cell) (Mariotti *et al.*, 2013). Data from the present study is in general agreement to that of Mariotti and colleagues (2013). It is acknowledged that experimental evidence for the exercise-induced DNA DSB-repair response is currently lacking, as such, conclusions are generally compared with radiation-induced data. DNA DSBs commonly result from the direct collision of radiation particles to the phosphodiester backbone and/or the hydrolysis of water, in turn creating multiple hydroxyl radicals in close proximity to the DNA architecture (Yamaguchi *et al.*, 2005); however, this is not the case in the present study. RONS-induced DNA damage typically results in single-strand damage and/or base oxidation with DNA DSBs as a function of exercise, occurring through two possible mechanisms: (1) the conversion of single-strand breaks, or 'clustered' lesions, to a DSB is a matter of probability, and relies on multiple single-strand breaks within 10^4 helical turns (Vilenchik & Knudson, 2003; Cannan & Pederson, 2017); (2) during the BER pathway, repair proteins attempt to seal the 1-2 nucleotide gap, however, in the instance of clustered lesions, the inefficient repair of single strand breaks on parallel strands can lead to the conversion of a DSBs (Eccles *et al.*, 2011). Mechanistically, the phosphorylation of H2AX by ATR/ATM serine/threonine kinases initiates the recruitment of proteins which determine the DNA DSB repair pathway; specifically, MRN complex, MDC1, BRCA1, and 53BP1 (Podhorecka *et al.*, 2010). Due to the activation of *γ*-H2AX in both the homologous recombination and non-homologous end joining repair pathways, it is important to employ the use of one, or more, colocalised repair/signalling factors associated with DNA DSB repair; in the case of the present

study, 53BP1 was used. 53BP1 is a key mediator/adaptor protein of the DNA damage response which becomes mobilised at the onset of a DNA double strand lesions; subsequently becoming hyperphosporylated (Anderson *et al.*, 2001; Rappold *et al.*, 2001). The validity of 53BP1 and *γ*-H2AX to determine double strand breaks in a colocalised manner has been confirmed across multiple experiments (Schultz *et al.*, 2000; Richie *et al.*, 2002; Sengupta *et al.*, 2004; Lee *et al.*, 2005). Within the present study, the increase in 53BP1 detection following exercise suggest the activation of the non-homologous end joining repair of DNA DSBs. To elaborate, 53BP1 promotes the non-homologous end joining repair pathway (Gupta *et al.*, 2013; Mirza-Aghazadeh-Attari *et al.*, 2019) and prevents homologous recombination repair by inhibiting BRCA1; a key regulator of the HR repair pathway (Bouwman *et al.*, 2010). Additionally, BRCA1 colocalises with H2AX, thus further promoting the activation of homologous recombination repair (Yoshida & Miki, 2004; Zhang, 2013). An overview of the DNA DSB repair-response and downstream protein interactions can be observed in Figure 5.8.

Figure 5.8 The DNA double strand break repair cascade and subsequent downstream interactions.



As this is the first study investigating the exercise-induced DNA damage-repair response (with specific interest of DSBs), the data presented provides insightful, yet limited, clarity on the mechanistic nature of exercise-induced DNA damage and repair pathways. Future research should incorporate the use of multiple repair and signalling factors to further elucidate the specific interaction of single- and double-strand damage as a function of exercise-induced oxidative stress.

5.4.3. General Oxidative Stress

Systemic indices of oxidative stress were further ascertained to elucidate the effect of exercise in hypoxia. Hypoxia *per se*, and to a greater extent exercise, caused an increase in lipid peroxidation aligned to a decrease in lipid-soluble antioxidant capacity; this data supports findings from similar studies (Davison *et al.*, 2006; Bailey *et al.*, 2018). Due to the presence of a double bond structure within PUFA side chains, biological membranes are a major target for RONS attack, causing hydrogen ion abstraction, thus, propagating lipid peroxidation. In the present investigation, a concurrent decrease in markers of lipid soluble antioxidants following exercise was evident, and further compounded by hypoxia. Alpha-tocopherol is an important chain-breaking antioxidant that attenuates the propagation phase of lipid peroxidation by reducing lipid peroxyl radicals; in turn, producing the α-tocopherol radical (Young & Woodside, 2001). Subsequently, α-tocopherol radicals are recycled during the univalent oxidation of ascorbate generating the ascorbyl radical. In the current study this process may be evident, whereby α-tocopherol concentration decreases while a simultaneous increase in the ascorbyl free radical is observed; this response was exacerbated in the hypoxic group. It has also been postulated that exercise may trigger the accumulation of intramuscular α-tocopherol by either selective mobilisation, or an increase in lipoprotein delivery for hydrolysis (Bailey *et al.*, 2007). Similarly, carotenoids exert antioxidant properties by holding an affinity for peroxyl radical intermediates. Thus, the observed decrease in lycopene and α-carotene may highlight their ability to protect lipid-rich structures against peroxidation as a function of high-intensity exercise. Although this change in lipid soluble antioxidants coincides with others (Sacheck *et al.*, 2003; Agulio *et al.*, 2003; Vierck *et al.*, 2012; Mrakic-Sposta *et al.*, 2015), it should be highlighted that some investigators have observed an increase or no change in antioxidants following exercise (McClean *et al.*, 2015; Brown *et al.*, 2018). Collectively, the observed reduction in lipid soluble antioxidants, and parallel

increase in DNA damage and base oxidation, supports the hypothesis that the cumulative effect of exercise and more so, hypoxia, leads to the generation of RONS, compromising endogenous antioxidant network (Fiedor & Burda, 2014). Hypoxia is a potent environmental stressor and is known to activate the accumulation of reducing equivalents at cytochrome c reductase and cytochrome oxidase within the mitochondrial electron transport chain (Bailey *et al.*, 2001). To expatiate, the superoxide anion is formed as ubisemiquinone, donates an electron to oxygen at site Q_i of cytochrome c reductase, which subsequently generates hydrogen peroxide, catalysed by Mn-SOD; this process can be compounded with hypoxia, and indeed, during exercise (Bailey *et al.*, 2009). Although not measured in the present study, the potential of reductive stress mechanisms to generate mitochondrial superoxide cannot be overlooked. It is possible mitochondrial superoxide, and downstream production of hydrogen peroxide and the hydroxyl radical, contributed to the observed changes in DNA damage and lipid peroxidation as a function of hypoxic exercise. Aside from the phenomenon of reductive stress, it is also likely that NO·, increased availability of catalytic free iron, and an increase in oxygen-centred free radical species from red blood cells contributed towards the hypoxic-induced lipid peroxidation observed. It should be noted however, lipid hydroperoxide analysis was confined to spectrophotometric detection in serum, and it is likely that the observed concentrations have been underestimated compared to the more sensitive HPLC detection method of plasma-based lipid hydroperoxides (Bailey *et al.*, 2018).

Ascorbyl free radical concentration following exercise was greater in hypoxia compared to normoxia, indicating an increase in free radical production via ascorbic acid oxidation (Bailey *et al.*, 2010). This coincides with observed lipid hydroperoxides, confirming that exercise increases the generation of free radical species. Further, although not directly quantified in the present study, oxygen-centred peroxyl radical production can increase following exercise (Davison *et al.*, 2008) and may be subsequently scavenged by ascorbic acid yielding a greater concentration of ascorbyl free radical species (Dendich *et al.*, 1986; Frei *et al.*, 1989). Determining the exact mechanism for ascorbyl radical production within this study is tangential, however it is possible that several radicals, including lipid peroxyl and alkoxyl radicals, could generate ascorbyl free radicals within the peripheral circulation ($R\cdot + AH- \rightarrow A\cdot- + R\text{-}H$) (Spasojevic, 2011). Additionally, the concentration of ascorbate within plasma is much greater than the reduction potential of any oxidising free radical with an affinity

for the ascorbyl free radical:ascorbate monanion couple ($E^{o\prime}$ = 282 mV); thus, the observed increase in ascorbyl free radical concentration provides evidence for oxidising free radical formation as a function of exercise in hypoxia (Bailey *et al.*, 2018). Due to the compartmentalisation of the ascorbyl radical in blood, it is conceivable that primary RONS generation from vasculature tissue involving NADPH oxidase, is the potential source of the observed increase in EPR signal intensity.

5.5. Conclusion

Exercise, and to a greater extent, exercise in severe hypoxia leads to DNA single- and double-strand damage, base oxidation, and lipid peroxidation; likely as a result of hydroxyl radical-mediated attack via the generation of superoxide and hydrogen peroxide. Concomitantly, a decrease in antioxidant capacity was observed across certain lipid-soluble antioxidants; potentially propagating structural damage to DNA. This study is the first to extensively characterise the DNA damage-repair response over 72-hours using multiple biomarkers of single- and double-stranded DNA damage following exercise in hypoxia and normoxia. In closing, this study provides justification for follow up work incorporating analysis of repair proteins to offer insight into the mechanistic nature of the exercise-induced double-strand DNA damage-repair response.

Chapter Six

Acute and Chronic Mitochondrial Targeted Quinone Administration and DNA Damage Following High-Intensity Intermittent Exercise

6.0 Abstract

Introduction: It is evident mitochondrial dysfunction can contribute to the pathology of several pathological conditions (Murphy, 2016); thus, highlighting the inherent importance of developing drugs/products which may reduce mitochondrial damage. This is the first study of its kind to determine the mechanisms of acute and chronic mitochondrial-targeted quinone supplementation on biomarkers associated with oxidative damage.

Methods: Twenty-four (*n*=24) apparently healthy, recreationally active males (age 25 ± 4 years, stature 181 ± 4 cm, mass 87 ± 11 kg), volunteered for the study, and subsequently provided their medical history prior to written informed consent. In a double blind, randomized, placebo-controlled design, participants were allocated to two groups; placebo (n=12) and MitoQ (n=12). To complete the experimental study, participants completed an acute (20mgs MitoQ or placebo 1 hour prior to exercise) and chronic (21 days of supplementation). During each phase, participants completed a single exercise trial of 4 x 4-minute bouts at 90-95% of HRmax with a 3-minute active recovery at 70% of HRmax.

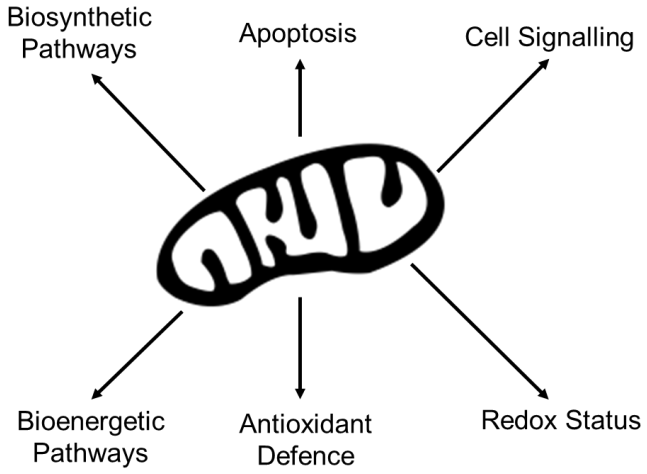
Results: During the acute and chronic phase, exercise significantly increased nuclear and mitochondrial DNA damage across lymphocytes and human muscle tissue ($P < 0.05$). Additionally, this was accompanied with significant changes in lipid hydroperoxides, the ascorbyl free radical, and α-Tocopherol ($P < 0.05$). MitoQ did not offer any prophylactic effect to any biomarker of oxidative stress during the acute phase. A protective effect of MitoQ was observed following chronic supplementation on nuclear DNA damage ($P < 0.05$, ES = 0.2), and mitochondrial DNA damage (lymphocytes; $P < 0.05$, ES = 0.29, muscle tissue; $P < 0.05$, ES = 0.38).

Conclusions: The data demonstrates that high-intensity intermittent exercise induces damage to the mitochondrial genome in both lymphocytes and muscle tissue. Moreover, a protective effect of chronic MitoQ supplementation on DNA damage was observed.

6.1 Introduction

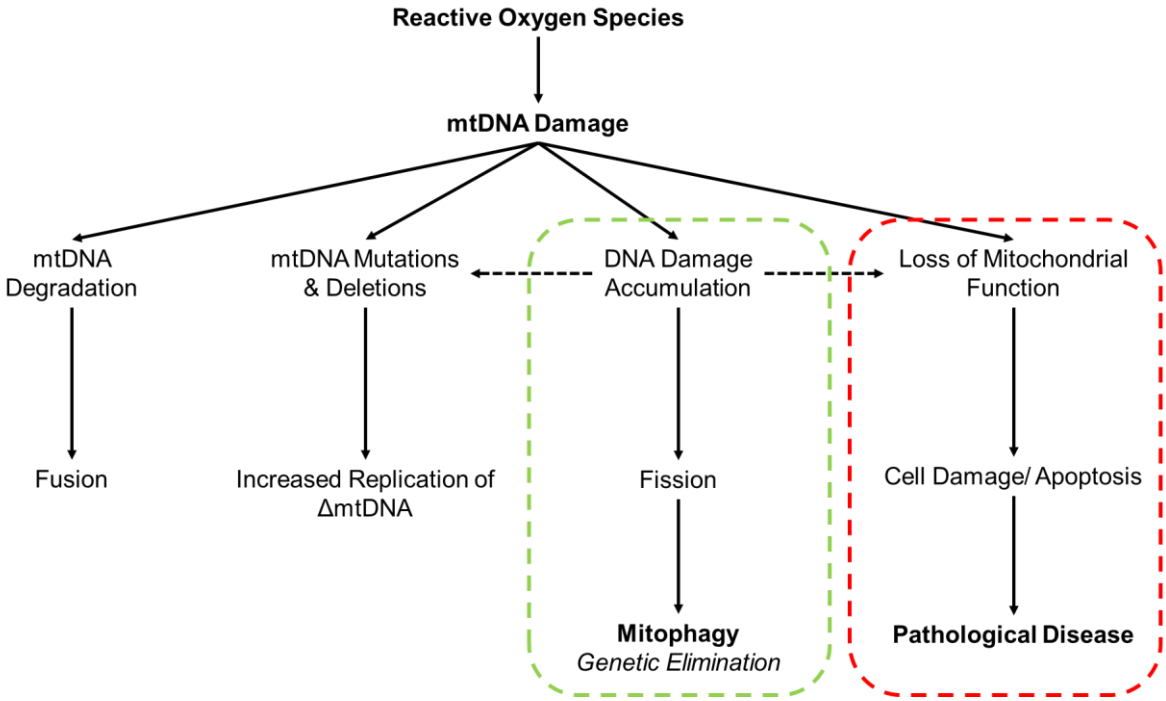
Mitochondria have an established role in the life and death of a cell; contributing to numerous cellular networks including energy production and biosynthetic pathways (Chan, 2012; Hoitzing *et al.*, 2015), but they also play an integral role in apoptotic and necrotic cell death (Murphy, 2016), as outlined below (Figure 6.1):

Figure 6.1 A summary of the major physiological functions associated with the mitochondria.



Although the mechanistic narrative may not be fully elucidated, the current body of evidence details a clear relationship between mitochondrial dysfunction and disease progression (Khan *et al.*, 2017; Chakrabarty *et al.*, 2018). Furthermore, the common denominator across the multitude of pathologies associated with mitochondrial dysfunction, is an increased generation of RONS, and subsequent mitochondrial DNA damage (Kadenbach *et al.*, 1995; Ozawa, 1995). It appears that this RONS-mediated, mitochondrial DNA damage initiates impairments in bioenergetics, apoptosis, cell proliferation, and inefficient ATP production; in turn, increasing the likelihood of impaired organ function and pathophysiology (Singh *et al.*, 2015; Van Houten *et al.*, 2016). Cellular responses and downstream effects of mitochondrial DNA damage are illustrated in Figure 6.2. This has led to investigations into the therapeutic potential of compounds which have demonstrated antioxidant properties, with specific interest in the restoration of mitochondrial health; including, MitoE (Mao *et al.*, 2010), tiron (Krishna *et al.*, 1992; Silveira *et al.*, 2003; Fang *et al.*, 2012), MitoC (Finichiu *et al.*, 2015), melatonin (Acuna Castroviejo *et al.*, 2011; Tan *et al.*, 2016), and lipoic acid (Lui, 2008; Ong *et al.*, 2013).

Figure 6.2. Cellular responses and downstream effects of mitochondrial DNA damage. The increased generation of ROS and/or loss of mitochondrial DNA repair can potentiate mitochondrial DNA damage and subsequent mitophagy of dysfunctional fragments. Green shading represents the physiological and protective responses to mitochondrial DNA damage. Red shading indicates pathophysiological responses to mitochondrial DNA damage; potentially leading to organ pathology and disease. Adapted from Van Houten et al., (2016).



One such therapeutic compound is the orally-available mitochondrial-targeted derivative of ubiquinone; Mitoquinone (MitoQ). Due to the covalency of triphenylphosphonium to quinone, MitoQ possesses the unique ability to accumulate within the mitochondria at approximately 100-1000x greater than non-mitochondrial targeted derivatives (Smith & Murphy, 2010). To elaborate, lipophilic cations increase their accumulation 10-fold for every 61.5 mV of membrane potential in accordance with the Nernst equation (Smith & Murphy, 2010); this effective uptake is also supported through the plasma membrane potential (Ross *et al.*, 2005). Once in mitochondria, MitoQ is absorbed into the inner membrane space and converted to the active antioxidant ubiquinol (Asin-Cayuela *et al.*, 2004; James *et al.*, 2007).

The antioxidant abilities of MitoQ have been shown to reduce lipid peroxidation in isolated mitochondria (Kelso *et al.*, 2001; Asin-Cayuela *et al.*, 2004) and attenuate peroxynitrite-mediated damage (James *et al.*, 2007). Other protective effects of MitoQ have been observed in disease models including cardiac ischemia/reperfusion injury (Adlam *et al.*, 2005; Neuzil *et al.*, 2007), chronic nitroglycerin exposure (Esplugues *et*

al., 2006; Graham *et al.*, 2009), sepsis (Supinski *et al.*, 2009), liver damage (Lowes *et al.*, 2008), and adriamycin exposure (Chandran *et al.*, 2009). The results achieved in these pathological conditions have solidified MitoQ as an attractive compound for a potential therapeutic treatment for certain human diseases. As such, there are multiple lines of research investigating the role of MitoQ supplementation in various clinical populations including Parkinson's disease (Snow *et al.*, 2010; Ghosh *et al.*, 2014), hepatitis C (Gane *et al.*, 2010), and aortic stiffness (Rossman *et al.*, 2018).

To date, no study has examined the effect of a targeted antioxidant on mitochondrial DNA damage following high intensity intermittent exercise (HIIE). Strenuous and/or prolonged exercise is known to produce an array of metabolic and physiological stress; in turn, potentiating disturbances in redox status, and damage to biological structures (Radak *et al.*, 2001; Senturk *et al.*, 2001; Pinho *et al.*, 2006). Exercise is known to cause a 10-20 fold increase in oxygen consumption, and 100-200 fold rise at the skeletal muscle level (Astrand *et al.*, 1986; Halliwell & Gutteridge, 2007). The increased oxygen consumption associated with exercise has been shown to increase the univalent reduction of oxygen to superoxide within the mitochondrial electron transport chain; specifically, at the NADH-coenzyme Q, succinate-coenzyme Q, and coenzyme QH2-cytochrome c reductase complexes (Silva *et al.*, 2009). Depending on the redox status of the cell, under physiological conditions this superoxide can increase the production of hydrogen peroxide, which diffuses from mitochondria, and act as a signalling molecule (Sena & Chandel, 2012; Quinlan *et al.*, 2013). Moreover, mitochondrially-derived superoxide can also propagate the generation of hydroxyl radicals, in turn increasing mitochondrial DNA damage and lipid peroxidation (Aguilo *et al.*, 2005; Sureda *et al.*, 2005). As such, the aim of this study is to investigate the prophylactic effect of a mitochondrial-targeted antioxidant, MitoQ, on exercise-induced mitochondrial DNA damage.

6.2 Materials and Methods

6.2.1. Human Participants

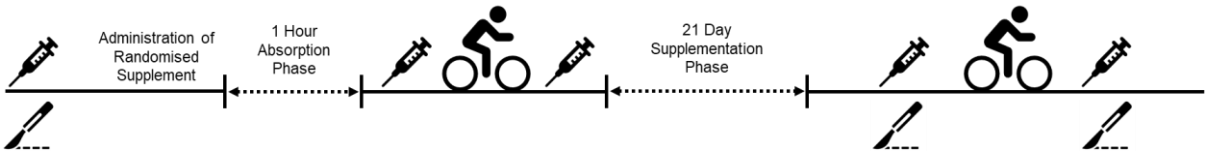
Twenty-four (n=24) apparently healthy, recreationally active males (age 25 ± 4 years, stature 181 ± 4 cm, mass 87 ± 11 kg), volunteered for the study, and subsequently provided their medical history prior to written informed consent. All participants were non-smokers and free from any form of medication or antioxidant supplementation for

4 weeks prior to, and throughout, the study. The study was conducted in accordance with the Declaration of Helsinki and approved by a local University Ethics Committee (REC/18/0018; See Appendix A).

6.2.2 Intervention Phases

In a double blind, randomized, placebo-controlled design, participants were allocated to two groups; placebo (n=12) and MitoQ (n=12). To complete the acute phase, the participants within the supplemental group consumed 20mgs MitoQ in a pill (Antipodean Pharmaceuticals; CA, USA), with the placebo group consuming visually identical pills, but excluding the active ingredient (Antipodean Pharmaceuticals; CA, USA) 1 hour prior to exercise. Upon completion of a high intensity exercise protocol, participants continued to supplement in their respective groups for a total of 21 days. A schematic overview of the experimental trail is depicted in Figure 6.3. Participants were instructed to consume MitoQ supplementation in a fasted state to maximise absorption based on known pharmacokinetic data (personal communication and unpublished data from Prof Michael Murphy and Dr Greg Macpherson, University of Cambridge).

Figure 6.3 Schematic overview of experimental study.



Participants remained in their assigned groups for the acute phase and the first 21 supplemental days of the chronic phase.

6.2.3 High Intensity Intermittent Exercise Protocol

For all experimental testing, participants were required to complete a standardised 12-hr overnight fast, and to refrain from exercise and alcohol consumption for 48 hours prior to testing. Following the familiarisation phase, participants completed an incremental test to exhaustion to determine maximum heart rate. Participants cycled at a cadence of 70-90 revolutions per minute on a friction-braked cycle ergometer to produce a power output equivalent to their bodyweight (1W/kg). The workload was increased by 0.5W/kg of body weight every 2 minutes until the participant could no longer maintain the required work rate (Jamnick *et al.*, 2018).

The HIIE trial consisted of 4 x 4-minute bouts. Each 4-minute work interval corresponded to 90-95% of HRmax with a 3-minute active recovery at 70% of HR_{max} as outlined by Helgerud *et al.* (2007). Continuous heart rate monitoring was achieved through the use of a portable short-angle telemetry device (Polar sports tester, Finland). Participants could drink water *ad libitum.*

6.2.4 Haematology and Muscle Sampling

Blood was extracted from a prominent antecubital forearm vein pre- and immediately post-exercise for both the acute and chronic phases. All blood was extracted using the vacutainer method, and subsequently centrifuged, aliquoted, and stored at -80°C prior to biochemical analysis. An exercise-induced haemoconcentration was determined using the equations of Dill and Costill (1974), incorporating haemoglobin and haematocrit indices. Packed cell volume (%) was measured using the microcapillary reader technique and corrected by 1.5% for plasma trapped within erythrocytes (Dacie and Lewis, 1968). In addition to blood samples, a subsample of participants provided skeletal muscle tissue (Placebo; *n*=5, MitoQ; *n*=5). Tissue was extracted from the baseline time point, and pre- and post-exercise time points of the chronic phase. Briefly, following local anesthesic (2% lidocaine), a small incision was made using a single use sterile scalpel (Swan-Morton, Sheffield, England). The biopsy device (Acecut biopsy needle, TSK laboratories, Soja, Japan) was then inserted at a 90-degree angle to the skin edge, and triggered to capture the muscle sample. The same investigator extracted all muscle tissue samples (Prof. Gareth Davison). Once collected, all samples were immediately flash frozen in liquid nitrogen and stored until subsequent analysis.

6.2.5 C2C12 Culture

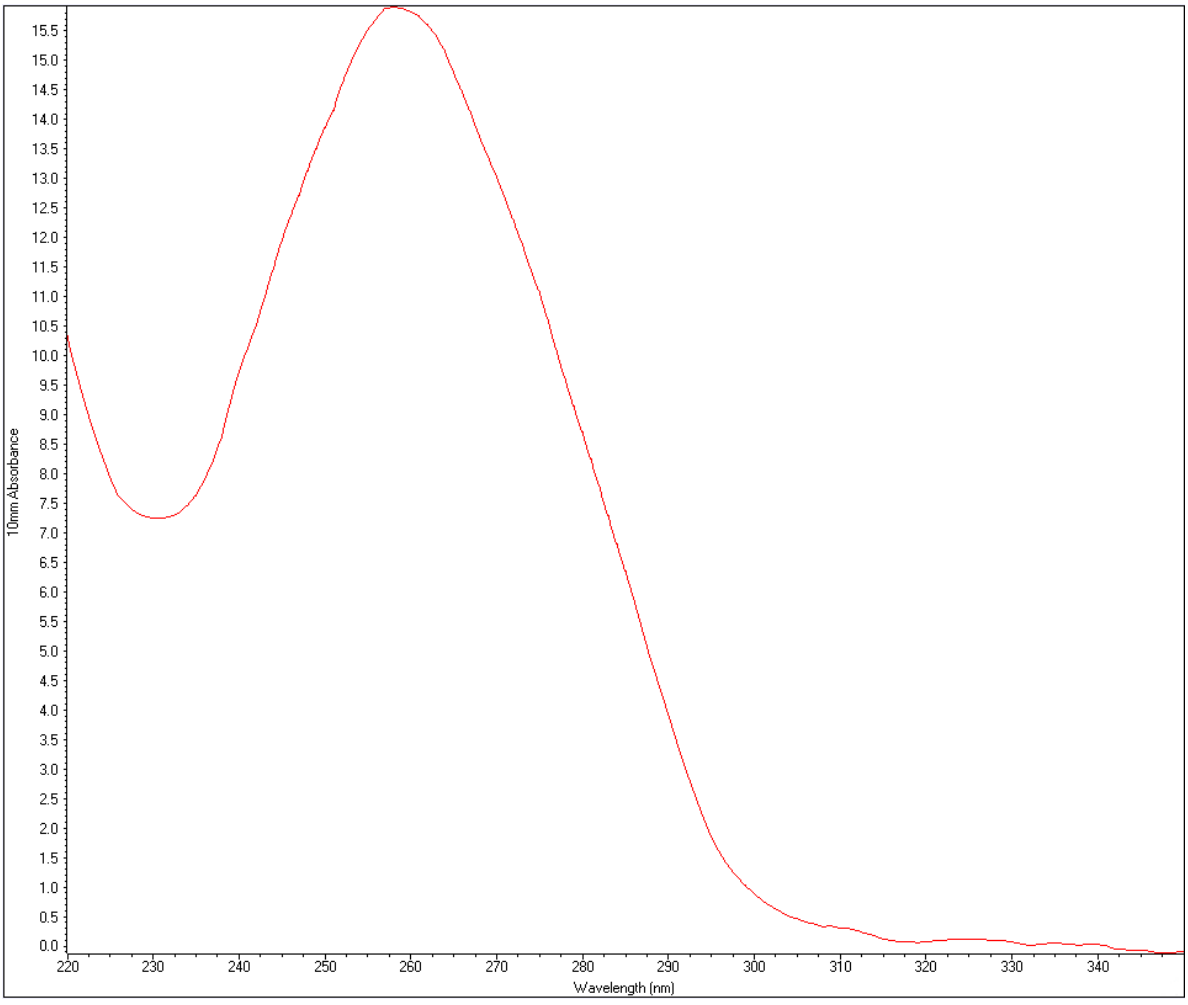
Mus Muculus C2C12 myoblasts (American Type Culture Collection, CRL-1772) were used to provide an insight to the mechanisms of oxidative damage to DNA by acting as a positive control. Cell cultures were grown as previously described (Kislinger *et al.*, 2005), in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 20% fetal bovine serum, 200 mM L-glutamine, 10 units/ml penicillin, and 10 µg/ml streptomycin at 37°C in an atmosphere of 5% CO_2. Adherent cells were harvested by trypsin incubation using 0.05% trypsin in EDTA (Gibco, USA) and seeded at a density of 1 x 10^5. Cell viability was assessed using the trypan blue exclusion assay (Sigma-

Aldrich), with all experimental cells exceeding ≥95% viability. All samples were incubated with exogenously applied hydrogen peroxide of an incrementally increasing concentration for a period of 30 minutes. This was performed at 4°C to attenuate DNA repair.

6.2.6 Long Amplicon-Quantitative Polymerase Chain Reaction

Total DNA was extracted from lymphocytes, human muscle tissue and mouse C2C12 cells using a Qiagen Genomic-Tip kit as previously published by Hunter *et al.*, (2011) and Furda *et al.*, (2015). Once successfully extracted, DNA quality and purity were quantified by using the Nanodrop spectrometry method (A_{260}/A_{280} ≥ 1.85) as outlined in section 3.6.2. A representative spectrophotometer is in Figure 6.4.

Figure 6.4 Representative nanodrop spectrophotometer from DNA samples following Qiagen Genomic Tip extraction.



DNA was quantified using PicoGreen as per the manufacturers instructions with flouresence measured with a 485 nm excitation filter and a 530 nm emission filter. Lambda DNA/HindIII was used to construct a standard curve in order to determine the concentration of unknown samples. All DNA samples were stored in TE buffer (10 mM Tris/1 mM EDTA) at 4°C.

To quantify cell mitochondrial DNA damage, the LA-qPCR method was used (Gonzalez-Hunt *et al.*, 2017; Saunders *et al.*, 2018). Further assay details are in section 3.6.4.

6.2.7. DNA Single Strand Breaks

DNA damage was measured in human peripheral blood mononuclear cells (PBMCs) using the single cell gel electrophoresis, or comet assay (Davison, 2016) as per section 3.4.1.1. The intra/inter-assay coefficients of variation (CV's) of this assay is <8%.

6.2.8 Lipid Hydroperoxides (LOOH)

Serum LOOH was measured spectrophotometrically using the method of Wolff (1994). A detailed description can be found in section 3.4.3. The intra/inter-assay coefficients of variation (CV's) of this assay is <5%.

6.2.9 Lipid Soluble Antioxidants (LSA)

LSA were analysed by simultaneous determination using the high-performance liquid chromatography (HPLC) method as per section 3.4.4. The intra/inter-assay coefficients of variation (CV's) of this assay is <7%.

6.2.10. Electron Paramagnetic Resonance (ESR) Spectroscopy

The ascorbyl free radical was measured using EPR on a Bruker EMX EPR spectrometer (Bruker Instruments Inc., Billerica, MA, USA). A full protocol is in section 3.4.2.1.

6.2.11 Statistical Analysis

The statistical package SPSS statistical software (IBM, Surrey, UK, v.25) was used for the statistical computation. Data was analysed using a two way repeated measures analysis of varience with $P < 0.05$ being deemed statistically significant. Following a significant interaction effect, between group differences were subsequently analysed

using a one-way ANOVA, while a Bonferroni paired samples t-test was used for within time differences. All data were represented as mean ± standard deviation with the expection of mitochondrial DNA damage; whereby, error bars represent mean ± standard error of the mean as recommended by Hunter *et al.*, (2011) and Furda *et al.*, (2015).

6.3. Results

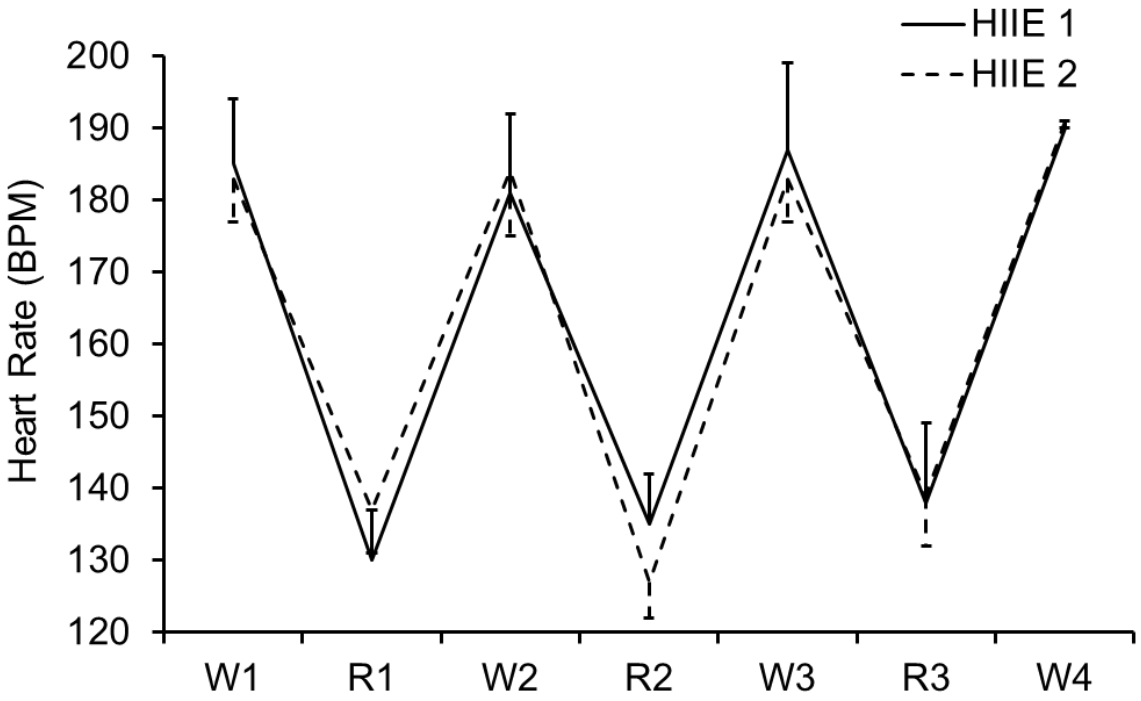
6.3.1 Compliance

Assessment of adherence was ascertained through two indirect measures to improve accuracy of reporting; pill/bottle counts and questioning the participant during laboratory visits (Spilker, 1992; Matsui, 2009). All 24 participants (100%) completed both the acute and chronic phases of the study. From a total of 528 administration opportunities (across all participants, and all phases), only 2 tablets were missed; thus, compliance was 99.6% for the entire study. No adverse side effects as a result of MitoQ consumption were reported during the supplementation period.

6.3.2 Exercise Performance Comparison

Physiological heart rate performance across the exercise trials, following acute and chronic supplementation, were similar with regards to exercise intensity ($P > 0.05$) as detailed in Figure 6.5. The maximum heart rate achieved during the initial incremental test to exhaustion was 196 ± 4 BPM.

Figure 6.5. Heart rates for the high-intensity intermittent trial across the acute and chronic phases. ***Note****: HIIE 1 refers to the exercise trial completed during the acute phase, whereas HIIE 2 refers to the chronic experimental phase as detailed in figure 6.3.*

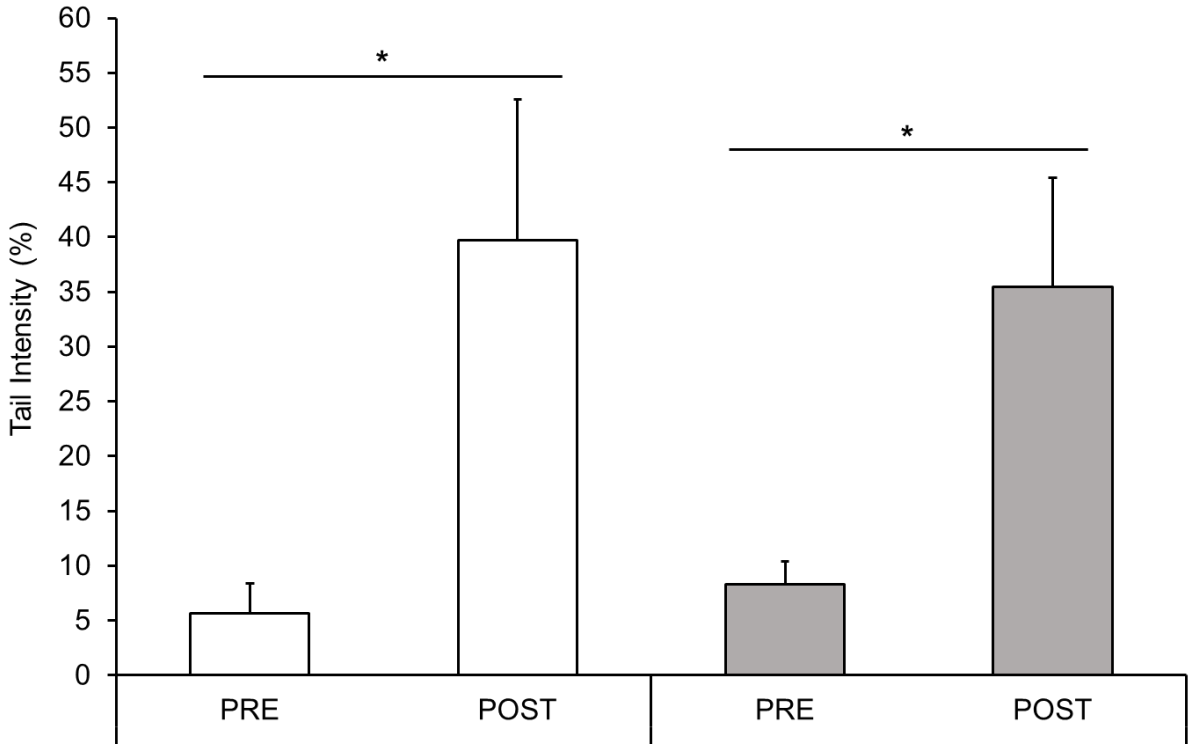


Performances across both trials were homogenous (P > 0.05).

6.3.3 Nuclear DNA Damage – Lymphocytes

Analysis of exercise-induced DNA damage following acute supplementation is depicted in Figure 6.6. There was an interaction effect for time × group ($P < 0.05$, ES = 0.4), and the post-hoc Bonferroni analysis indicating a difference between the pre- and post-exercise time points for each intervention group ($P < 0.05$, ES = 0.7, [Placebo = Δ34.1%; MitoQ = Δ27.1%]). Additionally, there was a main effect for time (pooled group pre- vs. post-exercise, $P < 0.05$, ES = 0.7). No interaction effect between groups was observed ($P > 0.05$).

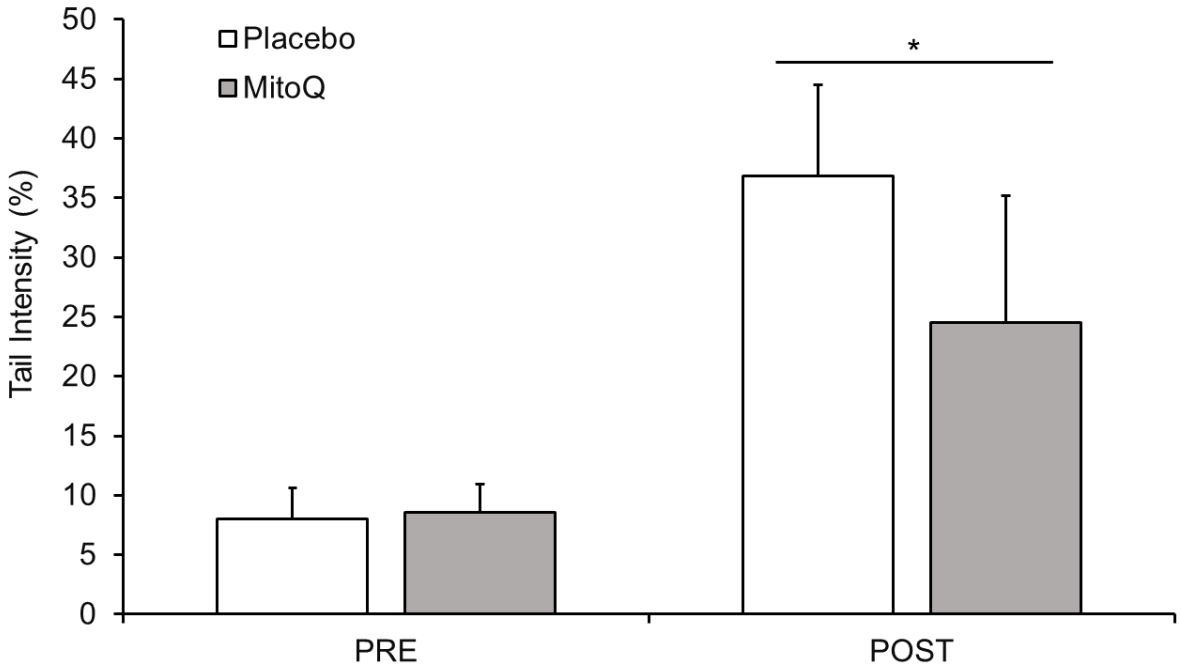
Figure 6.6. DNA damage following high-intensity intermittent exercise and acute MitoQ supplementation.



Data expressed as a mean ± standard deviation. represents a significant interaction effect (P < 0.05) within group.*

Following chronic supplementation (Figure 6.7), there was a significant interaction effect of time × group ($P < 0.05$, ES = 0.5), with post-hoc analysis indicating a difference between the placebo group and MitoQ group at the post-exercise time point ($P < 0.05$, ES = 0.2). Furthermore, there was a signifcant effect within groups from pre-exercise to immediately post-exercise ($P < 0.05$, ES = 0.4, [Placebo = Δ28.9%; MitoQ = Δ15.9%]).

Figure 6.7. DNA damage following high-intensity intermittent exercise and chronic MItoQ supplementation.

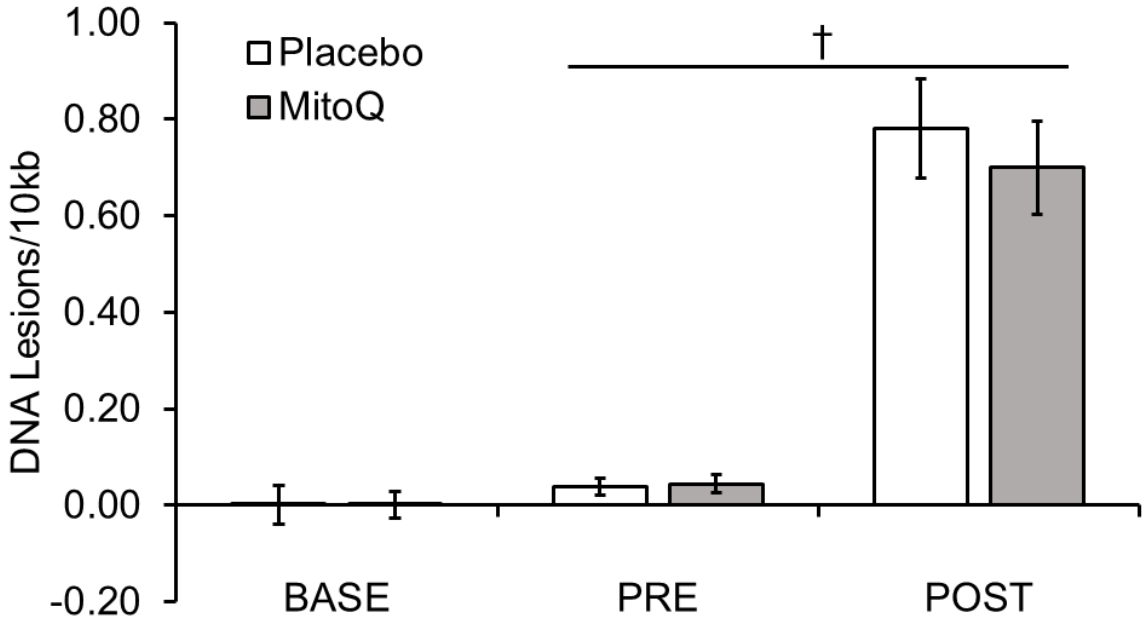


Data expressed as a mean ± standard deviation. represents a significant interaction effect (P < 0.05) between groups.*

6.3.4. Mitochondrial DNA Damage - Lymphocytes

The effects of acute MitoQ supplementation on lymphocyte mitochondrial DNA damage are presented in Figure 6.8. An increase in mitochondrial DNA damage ($P < 0.05$, ES = 0.53) was observed following high-intensity intermittent exercise. There was no difference between baseline and pre-exercise time points with regards to supplementation ($P > 0.05$), indicating that a single 20mg dose of MitoQ did not alter mitochondrial DNA integrity. No between group differences were observed between the pre- and post-exercise time points ($P > 0.05$), indicating acute supplementation of MitoQ does not provide protection against peripheral blood mononuclear cell mitochondrial DNA damage following exercise.

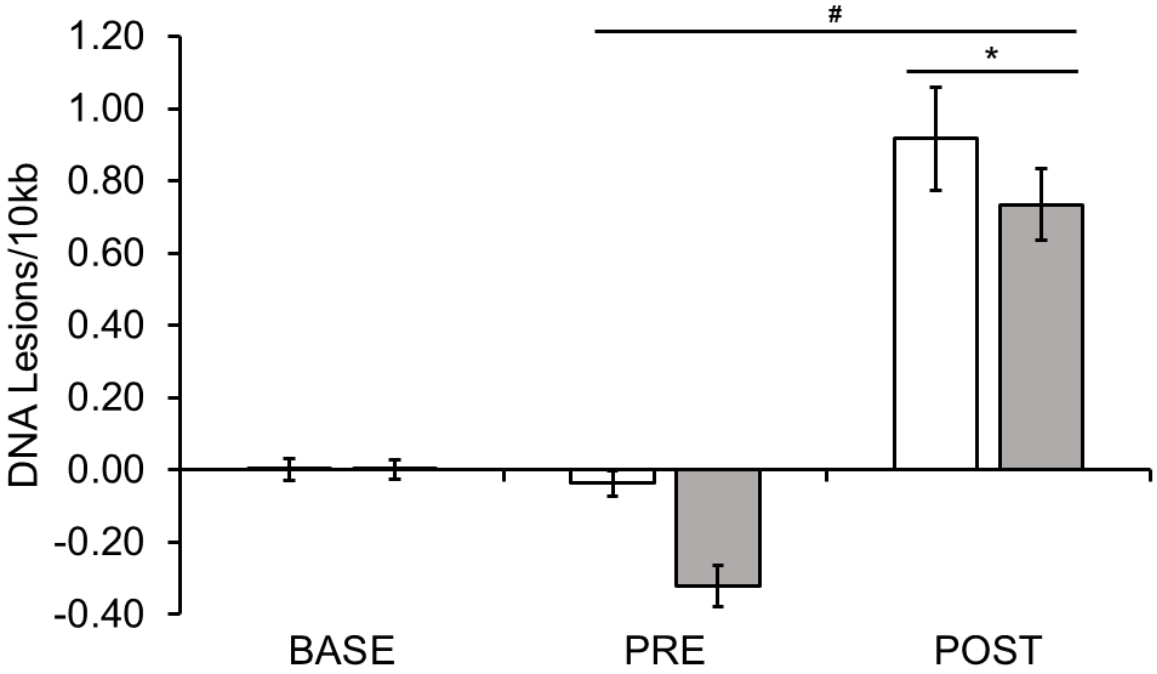
Figure 6.8. Mitochondrial DNA damage across the acute experimental phase.



Note: † indicates a main effect of time (P < 0.05).

The effect of high-intensity intermittent exercise and chronic MitoQ supplementation can be depicted in Figure 6.9. There was an increase in mitochondrial DNA damage as a function of exercise (pooled data; $P < 0.05$, ES = 0.53); it is plausible the observed damage is due to the generation of mitochondrial RONS. An interaction effect of time and group was also observed ($P < 0.05$, ES = 0.29); indicating MitoQ provided prophylaxis against high-intensity intermittent exercise following 21-days of supplementation.

Figure 6.9. Mitochondrial DNA damage across the chronic experimental phase.

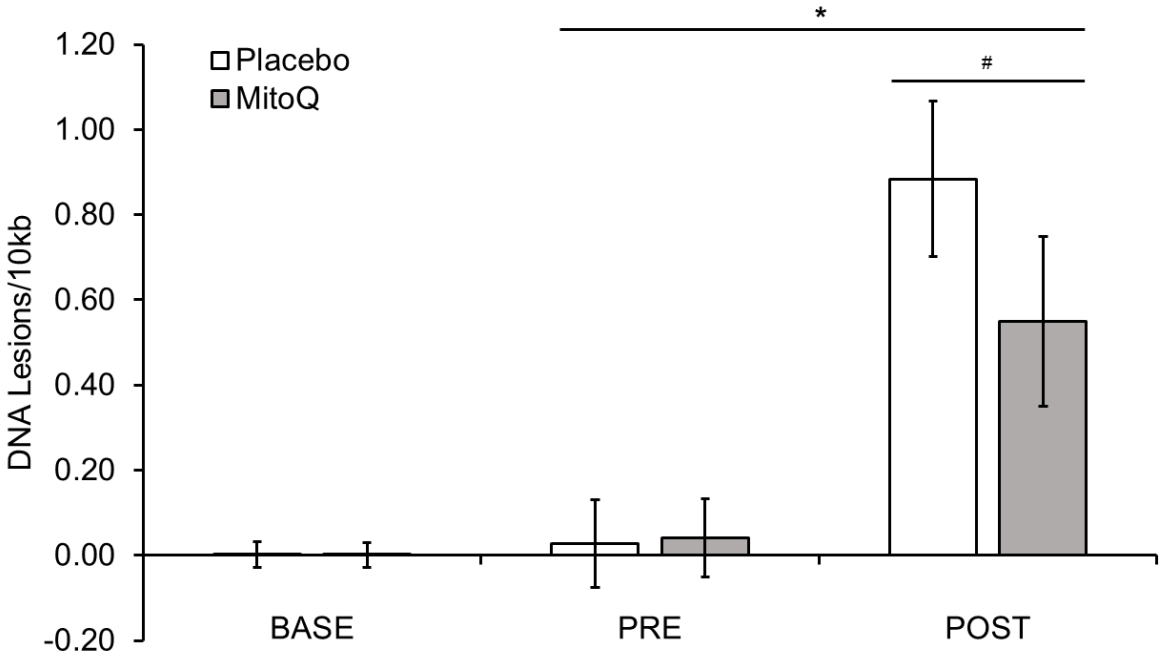


*Note: # indicates a main effect of time (P < 0.05). * represents a significant interaction effect of time x group (P < 0.05).*

6.3.5. Mitochondrial DNA Damage – Muscle Tissue

The effects of chronic MitoQ supplementation on mitochondrial DNA damage in human muscle are depicted in Figure 6.10. There was a significant interaction effect of time x group at the post-exercise time points ($P < 0.05$, ES = 0.38); indicating not only does high intensity intermittent exercise compromise DNA integrity, but chronic supplementation of MitoQ offers a prophylactic effect against exercise-induced mitochondrial DNA damage.

Figure 6.10. Muscle mitochondrial DNA damage across the chronic experimental phase.

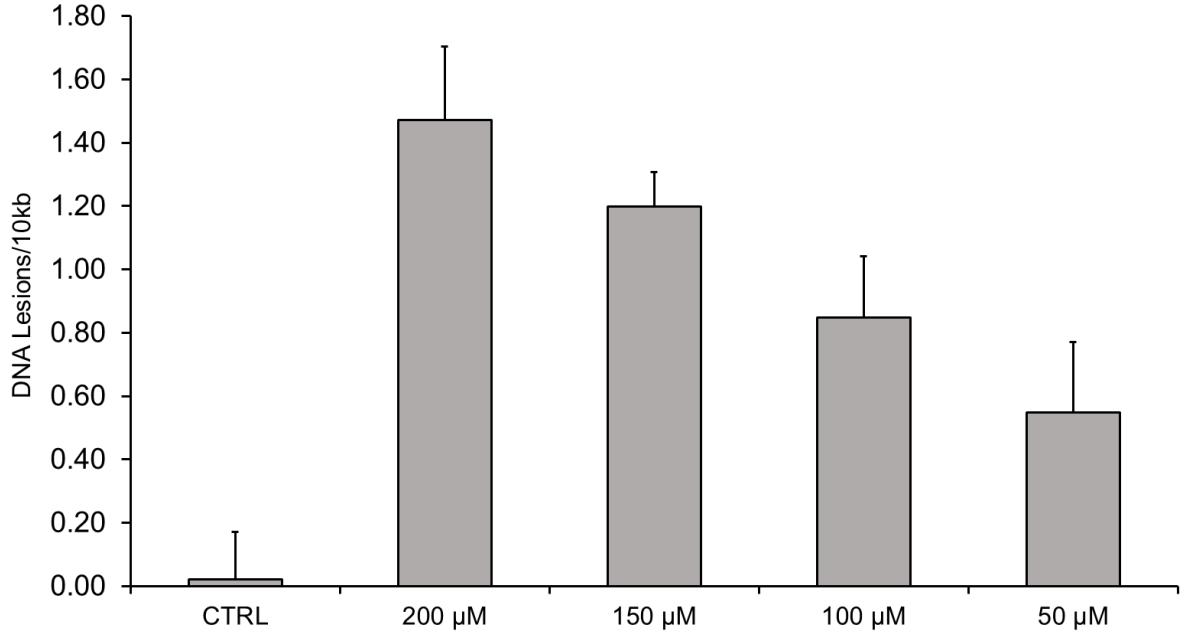


*Note: # indicates a main effect of time (P < 0.05). * represents a significant interaction effect of time x group (P < 0.05).*

6.3.6 Positive Control - C2C12 Mitochondrial DNA Damage

Mitochondrial DNA damage within C2C12 cells demonstrated a dose-dependent increase following exposure to hydrogen peroxide (Figure 6.11). The observed damage at 50-100µM is similar to that observed in lymphocyte and muscle cells.

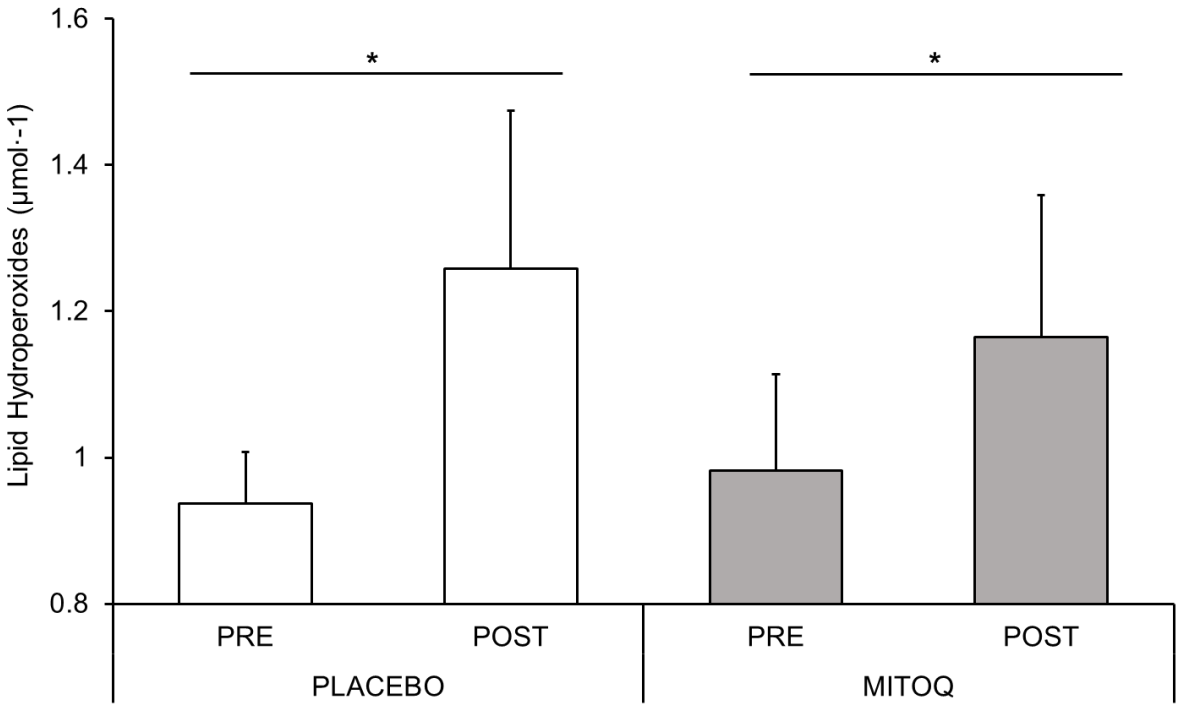
Figure 6.11. Hydrogen peroxide series on C2C12 mouse muscle cells; acting as positive control for LA-qPCR utilised in human muscle.



6.3.7 Lipid Hydroperoxides

There was no time x group interaction effect for lipid hydroperoxides following acute supplementation of MitoQ ($P > 0.05$, ES = 0.14) as depicted in Figure 6.12. However, lipid hydroperoxides increased in both placebo (Δ34%, $P < 0.005$), and supplement groups (Δ19%, $P = 0.029$) following exercise; indicating that high intensity exercise causes oxidative damage to lipids.

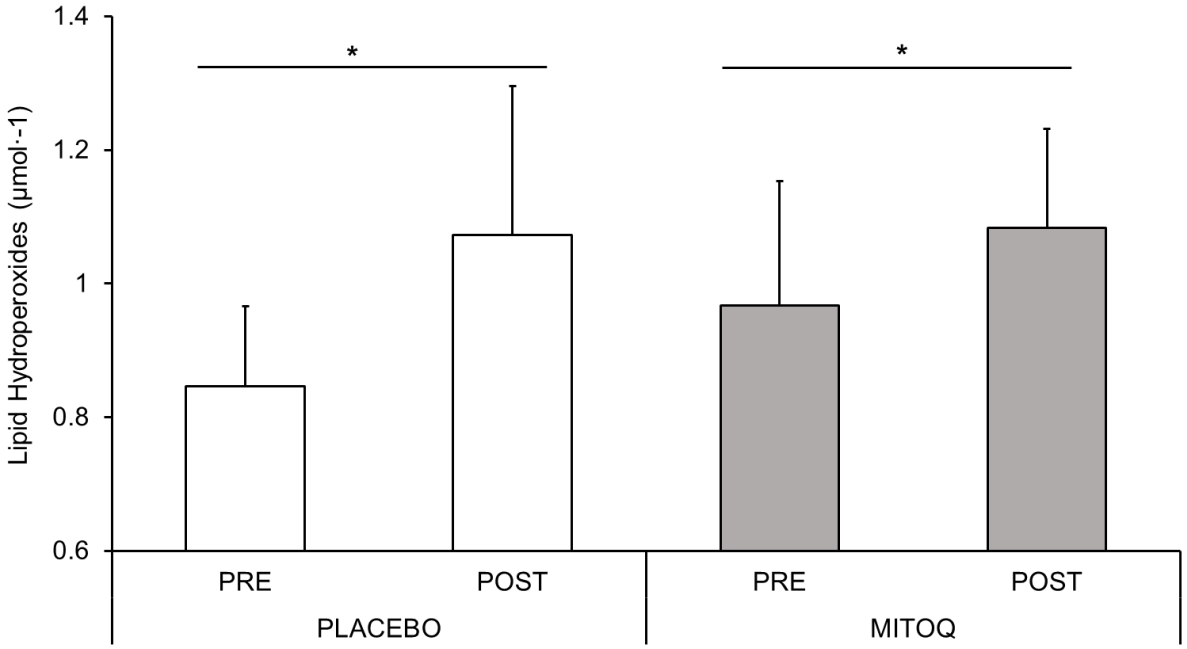
Figure 6.12. Lipid hydroperoxides following of high-intensity intermittent exercise across the acute phase.



*Note: * represents a significant within group effect (P < 0.05).*

There was no between group effect within the chronic phase (P > 0.05, ES = 0.21) as observed in Figure 6.13. However, there was a within group effect in both the placebo (Δ27%, P = 0.03) and MitoQ (Δ12%, P = 0.01) group; confirming the supposition that exercise induces lipid peroxidation. MitoQ offered no protective effect against exercise-induced lipid peroxidation.

Figure 6.13. Lipid hydroperoxides following high intensity intermittent exercise across the chronic phase.



*Note: * represents a significant within group effect (P < 0.05).*

6.3.8 Ascorbyl Free Radical

There was no time x group interaction effect of detected for the ascorbyl free radical ($P > 0.05$) following acute or chronic supplementation (Table 6.1). However, further analysis of pooled data (pre- versus post-exercise) indicated a main effect of time in both acute ($P = 0.02$) and chronic ($P = 0.03$) experimental trials.

Table 6.1. Acute and chronic exercise trials outlining ascorbyl free radical concentration following exercise. † represents a main effect of time (P < 0.05). Abbreviations – a.u. arbitrary units.

	Acute†		**Chronic†**	
	Pre-Exercise	**Post-Exercise**	**Pre-Exercise**	**Post-Exercise**
Placebo (a.u.)	*18948± 12099*	*25777± 12753*	*16839± 14088*	*29938± 20391*
MitoQ (a.u.)	*17038± 67331*	*27383± 14387*	*18293± 15982*	*30283± 15892*

6.3.9 Lipid Soluble Antioxidants

The data presented in Table 6.2. highlights the effects of acute and chronic MitoQ supplementation on lipid soluble antioxidants following high-intensity intermittent exercise. There was no interaction effect across any of the parameters ($P > 0.05$); however, further analysis indicated a main effect of time for *α*-tocopherol for both acute (pooled data; $P < 0.05$, ES = 0.1) and chronic (pooled data; $P < 0.05$, ES = 0.2) experimental phases.

*Table 6.2 Lipid soluble antioxidants at the pre-exercise and post-exercise time points for the acute and chronic trials. All values are presented as means ± SD and expressed as mmol·L^{-1}. * denotes a main effect of time (pooled data; p < 0.05).*

	Acute		**Chronic**	
	Pre-Ex	**Post-Ex**	**Pre-Ex**	**Post-Ex**
α-Tocopherol				
Placebo	18.13 ± 1.7	22.71 ± 3.5*	17.62 ± 1.2	22.27 ±3.2*
MitoQ	19.82 ± 0.9	22.97 ± 4.3*	19.21 ± 0.6	25.69 ± 4.6*
γ-Tocopherol				
Placebo	1.72 ± 0.2	1.83 ± 0.7	1.81 ± 0.6	1.93 ± 0.8
MitoQ	1.53 ± 0.4	1.41 ± 0.3	1.63 ± 0.3	1.81 ± 0.4
β-Carotene				
Placebo	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
MitoQ	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Xanthophyll				
Placebo	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
MitoQ	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
Retinol				
Placebo	1.76 ± 0.6	1.71 ± 0.9	1.88 ± 0.4	2.13 ± 0.6
MitoQ	1.83 ± 0.4	1.89 ± 0.6	1.69 ± 0.4	1.90 ± 0.8

6.4 Discussion

The aim of this study was to investigate the effect of high-intensity intermittent exercise on mitochondrial DNA damage, and subsequently ascertain if the mitochondrially-targeted antioxidant, MitoQ provides any prophylactic modification. The present study demonstrates that HIIE caused DNA damage to the nuclear and mitochondrial genomes, with mitochondrial DNA damage present in both peripheral blood mononuclear cells, and human muscle tissue. Additionally, supplementation of oral MitoQ provided a prophylactic effect against exercise-induced DNA damage; however, this effect was only observed following 21-days of supplementation, and not when MitoQ was administered 1-hour prior to a bout of HIIE.

6.4.1 Mitochondrial DNA Damage

It is evident from the previous studies that high-intensity exercise causes an increase in the production of RONS which may lead to an attack on cell integrity (Ortiz-Franco *et al.*, 2017); whereby the generation of superoxide and downstream production of the hydroxyl radical causes damage to biomolecules, including DNA (Powers & Jackson, 2008). The current study demonstrates a significant increase in both nuclear and mitochondrial DNA damage as a result of high-intensity intermittent exercise. These findings are consistent with others across a range of exercise modalities including rowing (Sardas *et al.*, 2012), running to exhaustion (Fogarty *et al.*, 2013a), and knee extension contractions (Fogarty *et al.*, 2013b; Gray *et al.*, 2014). The production of mitochondrial RONS is centrally controlled by core metabolic processes, and thus cannot be easily reduced without interfering with normal cellular function. It appears the damaging nature of mitochondrial-derived RONS originate from the overall persistent nature, and the ability of mitochondria to act as a net ‘sink’ as opposed to a net source of RONS (Starkov *et al.*, 2014; Andreyev *et al.*, 2015). Approximately 0.15% of the oxygen consumed by the mitochondria generates superoxide from single electron transfers from a redox donor to molecular oxygen (Quinlan *et al.*, 2013). The source of mitochondrial RONS is often attributed to the electron transport chain as a function of oxidative phosphorylation. In complex I, the NADH-oxidising site of the flavin, and the ubiquinone-reducing site are thought to be the two primary sources of superoxide production (Treburg *et al.*, 2011). With regards to complex III, the generation of superoxide is thought to be derived from the quinol oxidising site (Muller *et al.*, 2003; Kramer *et al.*, 2004; Quinlan *et al.*, 2011). Although these are the

predominant sites of mitochondrial RONS production, there are up to 11 different sites which contribute to total mitochondrial superoxide and hydrogen peroxide production (Quinlan *et al.*, 2012; Orr *et al.*, 2012; Perevoshchikova *et al.*, 2013); including $P66^{Shc}$, amine-oxidase, and α-glycerophosphate dehydrogenase (Tretter *et al.*, 2007a; Tretter *et al.*, 2007b; Mracek *et al.*, 2009). Regardless of the site of superoxide/hydrogen peroxide generation, the deleterious nature of mitochondrial RONS partially comes from the intracellular accumulation of transition/liable metals such as iron, which can undergo Fenton chemistry to produce hydroxyl radical species.

The undiscriminating nature of the hydroxyl radical is accountable for an array of oxidative mitochondrial DNA products, including 8-oxo-2′-deoxyguanosine, formamidopyrimidines, abasic sites, oxidised deoxyribose rings, aldehyde adducts, and/or base propenals (De Bont & van Larebeke, 2004; Trapp *et al.*, 2007). These DNA damage products (and their downstream counterparts), in combination with the oxidative nature of the mitochondrial matrix, allow mitochondrial DNA to be susceptible to oxidative attack more so than nuclear DNA (Richter *et al.*, 1988; Cline, 2012); furthermore, oxidative damage is more persistent in the mitochondrial genome (Akhmedov & Marin-Garcia, 2015). This supposition has been demonstrated across a multitude of exercise-based research studies (Jafari *et al.*, 2005; Parise *et al.*, 2005; Eluamai & Brooks, 2013). It has been postulated that this increased vulnerability could be explicated through a number of variables; for one, mitochondrial DNA lacks the protective nature of complex chromatin organisation and histone proteins (Dizdaroglu *et al.*, 1991; Ljungman *et al.*, 1992). Secondly, the mitochondria contain vicinal transition metal ions which can propagate the generation of the hydroxyl radical. Finally, the activation of secondary DNA- and lipid-oxidation products have the potential to cause further structural damage to the mitochondria (Niedernhofer et al., 2003; Del Rio et al., 2005). It is also worth highlighting that these secondary oxidation products have been shown to overwhelm the mitochondrial repair capacity (Yakes & Van Houten, 1997); including long- and short-patch base excision repair (Liu & Demple, 2010), de-alkylation (Cai *et al.*, 2005), components of mismatch repair (Souza-Pinto *et al.*, 2009), homologous recombination (Bacman *et al.*, 2009), and non-homologous end joining (Fukui & Moraes, 2009). Collectively, the data from the current study demonstrates an increase in mitochondrial DNA damage through the likely generation of superoxide and hydrogen peroxide; in turn, generating the hydroxyl radical in the presence of transition metals.

In support of the above supposition, the present work outlines the response of mitochondrial DNA damage to hydrogen peroxide incubation at various concentrations; with 200 µM inducing the largest amount of DNA damage. Additionally, these results are indicative of other investigators using similar *in vitro* models (Yakes & Van Houten, 1997; Ballinger *et al.*, 1999). It has been reported that hydrogen peroxide can induce up to 11 different base products (Jaruga & Dizdaroglu, 1996) which are expected to result in a block to the thermostable polymerase; based on work by Yakes & Van Houten (1997), it is likely the LA-qPCR assay is detecting the majority of lesions induced by the hydroxyl free radical. Han & Chen (2013) reported a similar dose-dependent response on mitochondrial DNA damage following hydrogen peroxide incubation at concentrations of 30 µM to 240 µM. It is plausible hydrogen peroxide could contribute to alterations in mitochondrial function, the redox potential of complex II, and mitochondrial membrane potentials; thereby increasing the susceptibility to oxidative damage (Ballinger *et al.*, 2000). Although not measured in the current study, it is likely that high concentrations of hydrogen peroxide could produce irreversible damage and inevitable cell death; this could be assessed through a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. Furthermore, future studies could incorporate the addition of mitoquinone mesylate into the cell culture media to examine the effects of MitoQ and mitochondrial RONS in C2C12 muscle cells.

6.4.2 Nuclear DNA Damage

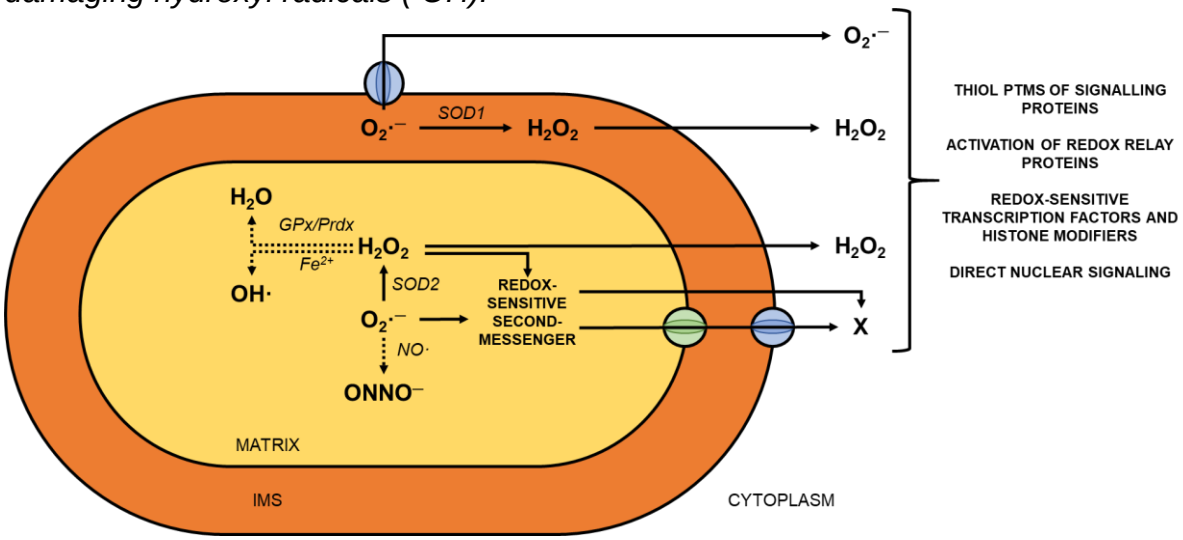
Initially reported by Hartmann *et al.* (1994), high-intensity, strenuous exercise is known to cause DNA damage. Consistent with this, others have confirmed this notion when exercise intensity approaches $\dot{V}O_{2max}$ (Davison *et al.*, 2005; Fogarty *et al.*, 2011; Williamson *et al.*, 2018). The current study demonstrates a significant increase in nuclear DNA damage as a result of high-intensity intermittent exercise as assessed by the comet assay. Furthermore, similar concentrations of oxidative DNA damage were observed in both the placebo and supplement groups during the acute phase. This supposition was confirmed during the chronic phase in the placebo group, indicating the ability of high-intensity intermittent exercise to cause structural changes to DNA; some of which, were mitigated by chronic ingestion of MitoQ. As outlined by Cobley *et al.* (2015a), exercise increases the generation of superoxide and hydrogen peroxide while simultaneously disrupting transition metal handling; in turn, increasing the

likelihood of hydroxyl radical generation due to the presence of liable iron and copper (Close *et al.*, 2005). As a result, the hydroxyl radical reacts with DNA bases at diffusion-controlled rate ($k \sim 5–8 \times 10^9\ M^{-1}\ s^{-1}$ for guanine [Chatgilialoglu *et al.*, 2011]); potentially generating other end products which can further propagate oxidative damage (Dizdaroglu, 2012). Although the present study corroborates the conclusions regarding exercise and DNA damage (Tryfidou *et al*., 2019), it is important to outline the discrepancies between nuclear and mitochondrial DNA damage.

Although the production of the hydroxyl radical may be derived from several mitochondrial and non-mitochondrial sources (as outlined in Chapter 2), the damaging nature of the hydroxyl radical requires it to be generated proximally to DNA (Halliwell, 2012). Mitochondria have a greater ability to generate hydrogen peroxide due to a greater dismutation capacity, compared to the nucleus (Murphy, 2009; 2012). As a result, mitochondrial hydrogen peroxide may indeed react with labile transition metals or heme/iron sulphur centred proteins as a function of exercise, thereby inducing oxidative damage to lipids or DNA (Collins *et al.*, 2012). On the contrary, nuclear DNA damage likely requires diffusion of hydrogen peroxide in close proximity to the nuclear genome (Cobley *et al.*, 2015); although this likely occurs from other cellular organelles (such as the endoplasmic reticulum, or membrane-bound NADPH oxidases), the mitochondria may also contribute to nuclear DNA damage. For one, as outlined by Murphy (2012), and summarised in Figure 6.14, mitochondrial hydrogen peroxide generation has a large capacity for diffusion and is hypothesised to play distinct roles in downstream physiological signalling responses; including post-translational modifications (Finkel, 2012; Go *et al.*, 2015). This retrograde signalling from the mitochondria to the nuclear domain may account for the potential of mitochondrial hydrogen peroxide to impact nuclear DNA damage (Cobley *et al.*, 2015). It should be noted however, that the diffusion mechanism of mitochondrial hydrogen peroxide, whether free or facilitated, remains equivocal and was not measured in the current study. With that being said, the data in the present study demonstrates significant DNA damage to the nuclear genome following exercise, which is likely derived from non-mitochondrial RONS sources. To corroborate this supposition, Gonclaves and colleagues (2015), provides evidence for the rates of mitochondrial superoxide and hydrogen peroxide production. They estimate that the greatest rate of hydrogen peroxide production and percentage of electron leakage is under resting conditions; these variables became incrementally lower as exercise intensity increases as the

redox centres that donate electrons to oxygen become more oxidised. Although these findings were drawn from *ex vivo* experiments, it suggests mitochondria are unlikely to contribute to the overall increase in RONS production as a result of exercise; thus, supporting the hypothesis by others that NADPH oxidase play an integral role in exercise-induced oxidative stress (Sakellariou *et al.,* 2013; Sakellariou *et al.,* 2014).

Figure 6.14. A simplistic schematic of mitochondrial RONS generation and downstream implications for cell signalling. Superoxide is generated on both sides of the inner mitochondrial membrane and hence arises in the matrix or the intermembrane space (IMS). Superoxide can be converted to hydrogen peroxide by SOD1 in the IMS or SOD2 in the matrix. The resulting hydrogen peroxide can cross membranes and enter the cytoplasm to promote redox signalling. Superoxide is not readily membrane permeable but may be released into the cytoplasm through specific outer membrane channels. In addition to signalling in the cytoplasm directly, both superoxide and hydrogen peroxide could, in principle, oxidize or modify other molecules in mitochondria that can be released/exposed to the cytoplasm to signal (redox-sensitive second messenger; X). These mitochondrial RONS (mtRONS) can generate signalling responses and changes in nuclear gene expression in multiple ways. There are other fates of mtRONS that would prevent signalling (or potentially enact other signalling and damage responses). For example, superoxide can react with nitric oxide (NO·) to form peroxinitrite (ONOO−). This would prevent its conversion to hydrogen peroxide, could cause damage by the highly reactive peroxynitrite, and could potentially limit NO availability for its own type of signalling. Hydrogen peroxide can be eliminated enzymatically by glutathione peroxidase (Gpx) in the matrix or peroxiredoxins (Prdx) in the matrix and elsewhere in the cell. Peroxiredoxins can also promote redox signalling by promoting disulfide bond formation in target proteins. Finally, in the presence of transition metals, hydrogen peroxide can generate damaging hydroxyl radicals (·OH).



6.4.3 Prophylactic Effect of MitoQ

Acute supplementation of 20mg of MitoQ 1-hour prior to exercise, had no effect on mitochondrial or nuclear DNA damage. With that being said, following 21-days of chronic supplementation, there was a reduction in mitochondrial DNA damage in peripheral blood mononuclear cells and human muscle tissue; this also coincided with a reduction in single-strand damage to nuclear DNA as quantified by the comet assay. Although considerable evidence has indicated that exercise increases DNA damage (Tryfidou *et al.,* 2019), the effect of mitochondrial-targeted antioxidants on mitochondrial RONS and resulting oxidative damage, remains an active area of research.

To the best of the author's knowledge, this is the first study to investigate MitoQ supplementation on oxidative damage to DNA in humans. The current study indicates that acute supplementation of MitoQ 1-hour prior to exercise had no effect on any measure of DNA damage; irrespective of tissue type. As reviewed in Table 2.5, there is a scarcity of human studies (especially in healthy, exercising populations) on the appropriate dosage to induce a prophylactic effect; thus, extrapolation of a human recommendation to animal studies is difficult. In addition, the lack of prophylaxis may be explained by the oral consumption and first pass metabolism of the stomach and liver (Pond & Tozer, 1984); as a result, the rate and amount of supplement reaching systemic circulation within the hour absorption phase will be reduced. Although bioavailability and tolerance of MitoQ has been clarified *in vivo* (Smith & Murphy, 2010), an appropriate dose and delivery to the site of action have yet to be fully elucidated within mitochondria of human participants (Braahuis *et al.,* 2018); this may be elucidated by pharmacokinetic analysis.

With that being said, chronic MitoQ supplementation for 21-days provided a protective effect to mitochondrial DNA in PBMCs and human muscle following a bout of high-intensity intermittent exercise. Mitochondrial DNA damage was assessed by LA-qPCR under the basis that amplification of a long segment of mitochondrial DNA (~10 kbp) will be more affected than a short segment (~200 bp); in turn, the relative amplification indicating the extent of mitochondrial DNA damage (Gonzalez-Hunt *et al.,* 2017; Saunders *et al.,* 2018). Ng and colleagues (2014) observed a reduction in mitochondrial DNA damage in *C elegans* treated with MitoQ, in addition to offering protection to complexes I and IV of the electron transport chain; these findings are consistent with others (Dare *et al.* 2015; Hu *et al.*, 2018). Similarly, Brand and

colleagues (2018) report the use of targeted antioxidants to positively attenuate UV-induced mitochondrial DNA damage. The protective effects (against DNA damage) of chronic MitoQ supplementation on PBMCs and muscle tissue observed in the present study may be attributed to a reduction in mitochondrial RONS formation; however, the data from the present study does not allow for the necessary insights to identify the specific mechanisms at work. Additionally, it has also been reported that MitoQ administration may attenuate mitochondrial DNA damage via NRF2 signalling, in turn, upregulating antioxidant genes such as HO-1, NQO-1 and γ-GCLC (Hu *et al.*, 2018), thereby providing protection against free radical formation and oxidation; however, gene expression was not an outcome measure in the present study, and this latter point remains speculation at this juncture.

In addition, the high prevalence of vulnerable mitochondrial lipids (such as anionic cardiolipin, phosphatidylethanolamine, and phosphatidylcholine; Schlame & Greenberg, 2017), potentiates RONS-mediated peroxidation which can further promote mitochondrial dysfunction; specifically, changes to lipid membrane fluidity, oxidation of protein complexes, inactivation of membrane-bound proteins, and inducing mitochondrial DNA damage (Wong-ekkabut *et al.*, 2007; Ademowo *et al.*, 2017). Consequently, these changes have been shown to promote mitochondrial DNA mutations, respiratory chain dysregulation, and a reduction in enzymatic antioxidant capacity (Simoncini *et al.*, 2015). It is highly plausible that the reduction in mitochondrial DNA damage following chronic MitoQ supplementation was a result of reduced RONS-induced modifications to mitochondrial-derived lipids; in turn, decreasing the likelihood of generating secondary lipid radicals which have the capacity to damage mitochondrial DNA.

6.4.4. Ancillary Measures of Oxidative Stress

The data from the current study supports the notion that high-intensity intermittent exercise promotes oxidative stress; as evident by the changes in lipid peroxidation, ascorbyl free radical formation, and α-tocopherol concentration. Furthermore, neither acute nor chronic MitoQ supplementation provided any protective effect against the observed exercise-induced oxidative stress.

Lipid peroxidation is a RONS-induced deleterious process by which polyunsaturated fatty acids become oxidised specifically at the carbon double bonds,

and the ester connection between the fatty acid and glycerol (Fernandez-Moriano *et al.*, 2017); consequently, this process is propagated by unstable, adjacent lipid radicals. With these oxidative products being recognised in aging and age-related diseases (Negre-Salvayre *et al.*, 2010), it is unsurprising that lipid peroxides are gaining more attention as potent, enduring, and physiologically relevant molecules of oxidative stress (Poli *et al.*, 2008; Mattson, 2009). Additionally, due to the physiological functions associated with the mitochondria, RONS-induced mitochondrial damage has a direct impact on alterations in cellular bioenergetics, specifically lipid dysregulation (Tindale *et al.*, 2017); not to mention the potential to exacerbate mitochondrial RONS production, and the detrimental downstream consequences of such reactions (Ademowo *et al.*, 2017).

Irrespective of supplementation group (acute or chronic), the present study demonstrated an increase in lipid peroxidation following high-intensity intermittent exercise. This exercise-induced modification to lipid membranes has been repeatedly demonstrated by others across a multitude of exercise durations, intensities, and modalities (Lovlin *et al.*, 1987; Kanter *et al.*, 1993; Leaf *et al.*, 1997; Davison *et al.*, 2002; Viitala *et al.*, 2004; Michailidis *et al.*, 2007; Goldfarb *et al.*, 2007). Several investigators have also demonstrated an attenuation of exercise-induced lipid peroxidation following supplementation of vitamin E (Dillard *et al.*, 1978; Schneider, 2003), ascorbic acid (Sastre, 1992; Goldfarb, 2005), NAC (Sen, 1994; Medved, 2004), and uric acid (Waring, 2003); similarly, others have corroborated these findings by using antioxidants in combination (Bryant *et al.*, 2003; Tauler *et al.*, 2006; Bloomer *et al.*, 2006; Goldfarb *et al.*, 2007).

It is reasonable to propose that administration of mitochondrial-targeted antioxidants could possibly contribute to restoration of mitochondrial integrity (and functionality), by reducing the deleterious effects of RONS; thus, alleviating biological ‘footprints’ associated with oxidative damage (Cai *et al.*, 2015; Zhang *et al.*, 2016). This is evident in clinical populations as oral and/or intraperitoneal administration of MitoQ has been used as a therapeutic treatment in Alzheimer’s disease, sepsis, fatty liver disease, and type I diabetes (Gane *et al.*, 2010; Snow *et al.*, 2010; Smith *et al.*, 2011); these findings are supported by others (Ma *et al.*, 2015; Hao *et al.*, 2016; Reddy *et al.*, 2017). Although numerous mitochondrial therapeutics exist, currently MitoQ has demonstrated the greatest efficacy; especially within clinical populations (Kelso *et al.*, 2001; Smith *et al.*, 2003; Adlam *et al.*, 2005; Esplugues *et al.*, 2006; Smith *et al.*, 2011).

Although the efficacy of general antioxidant supplementation and exercise-induced oxidative stress has been well characterised, there is a scarcity of literature surrounding mitochondrial-targeted antioxidants in exercise. To the best of the author's knowledge, Shill and colleagues (2016) were the first to examine the effects of MitoQ on endurance exercise training; specifically examining skeletal muscle capacity, maximal oxygen uptake, and circulating angiogenic cells. Despite different methods employed for the quantification of lipid peroxidation, neither Shill *et al.* (2016) nor the results of the present study demonstrated a prophylactic effect of MitoQ supplementation. Mechanistically, there are a number of physiological variables which may elucidate these findings. For one, the hydrophilic nature of MitoQ bound to the positively charged triphenylphosphonium allows for the preferential accumulation of the compound to increase by several hundred-fold within the mitochondria (Smith & Murphy, 2007; Fujimoto & Yamasoba, 2019). Once bound to the matrix-facing surface of the inner-mitochondrial membrane, it is continually recycled to the active antioxidant ubiquinol by succinate:ubiquinone oxidoreductase (Sakellariou et al., 2016). With respect to the current study, lipid hydroperoxides were determined within serum, indicating lipid peroxidation of vascular cell membranes; thus, lacking the sensitivity and/or specificity to distinguish between mitochondrial and non-mitochondrial sources of lipid peroxidation. It is also physiologically plausible that MitoQ administration did reduce mitochondrial lipid peroxidation as a function of high-intensity exercise; indeed, the reduction in mitochondrial DNA damage observed from MitoQ supplementation suggests a reduction in damaging mitochondrial RONS. With that being said, this is purely speculative, and future research should incorporate measures of lipid peroxidation which are specific to isolated mitochondria; this method has been used successfully by others (Willis, 1966; Kaur *et al.*, 2007; Wani *et al.*, 2011). It is also important to highlight in recent years mitochondria have been criticised as the major source of RONS (Urso & Clarkson, 2003), with accumulating evidence suggesting the primary sources of exercise-induced RONS being NADPH- and xanthine-oxidase (St-Pierre *et al.*, 2002; Jackson *et al.*, 2007; Powers & Jackson, 2008). Especially in the context of high intensity exercise, it is hypothesised that plasma xanthine oxidase is required for activating the exercise adaptation response within skeletal muscle (Vina *et al.*, 2000; Gomez-Cabera *et al.*, 2005).

Collectively, the current study suggests that high-intensity intermittent exercise leads to mitochondrial and nuclear DNA- and lipid-damage. The presence of the

ascorbyl free radical post-exercise indicates that strenuous exercise generates free radicals which have the potential to damage biomolecules. The simultaneous increase in DNA damage and lipid peroxidation, in tandem with the presence of ascorbyl free radical suggests that exercise increases the generation of RONS (Davison *et al.*, 2006). For one, Davison *et al.*, (2006) demonstrated a clear increase in free radical concentration using EPR spectroscopy following a single bout of moderate aerobic exercise; this was further confirmed following exhaustive exercise (Davison *et al.*, 2008), and later followed by Fogarty and colleagues (2011), who demonstrated a positive correlation between lipid-derived alkoxyl free radicals, and oxidation of DNA and lipids. It was also concluded that primary or secondary free radicals generated during the peroxidation cascade have the potential to attack DNA. Although alkoxyl free radicals were not measured in the present study, the increase in lipid peroxidation and DNA damage presents a viable mechanism for the observed data. The presence of the ascorbyl free radical suggests that ascorbic acid is oxidised to potentially scavenge other more potent free radical species (Piloni & Puntarulo, 2016). For example, as indicated by Davison *et al.* (2008), exercise generates oxygen-centred peroxyl radicals which may be scavenged by ascorbic acid; thus, increasing the concentration of the ascorbyl free radical. The current study does not allow for differentiation between the mechanism of actions; however, it is plausible that several reactive species are responsible for ascorbyl free radical generation including, superoxide, hydroxyl, and lipid-derived alkoxyl and peroxyl radicals (Spasojevic, 2011). Due to the use of EPR analysis within plasma from peripheral circulation, it is likely the primary free radicals are generated via NADPH-oxidase (Munzel *et al.*, 2005); however, additional mechanisms may include xanthine oxidase (Zweier & Talukder, 2006), and arachidonic acid enzymes (Shyu *et al.*, 2014).

Interesting, the current study also observed an increase in α-tocopherol concentration following exercise. Others report similar changes in systemic concentrations of lipid soluble antioxidants following high intensity exercise (Fogarty *et al.,* 2013). Alpha-tocopherol is a chain-breaking antioxidant and an important terminator of the propagation of lipid peroxidation (Qing & Ames, 2002); however, neither the lipid peroxidation data or the alpha-tocopherol indicate that this is occurring in the present study. The observed increase in alpha-tocopherol in response to high-intensity exercise could be due to the release of membrane-bound antioxidants into peripheral vascular circulation as a result of lipolysis and fatty acid mobilisation

(Pincemail *et al.,* 1988; Davison *et al.,* 2002). Further, it has be suggested that this response may compounded from exercising in a fasted state (Long *et al.,* 2008). The increase in oxidative damage within PBMC (nuclear and mitochondrial DNA), skeletal muscle tissue (mitochondrial DNA), and corresponding changes to lipid hydroperoxides, lipid soluble antioxidants, and the presence of the ascorbyl free radical, potentially indicates a systemic increase in a number of reactive species.

6.5 Conclusions

This is the first study to examine the effect of MitoQ on exercise-induced oxidative damage to DNA. The data demonstrates that high-intensity intermittent exercise induces damage to the mitochondrial genome in both lymphocytes and muscle tissue. These observations were accompanied by an increase in nuclear DNA damage. Moreover, a protective effect of chronic MitoQ supplementation on DNA damage was observed; it is plausible this could be explained through the free radical-scavenging properties of MitoQ, or indeed via the attenuation of secondary lipid peroxides which could potentiate the DNA damage response. These findings were not corroborated in the acute supplementation group, and this may be due to an insufficient 1-hour absorption period to allow the MitoQ to pass through the gastrointestinal tract and accumulate within the mitochondrial of the target cells. Furthermore, there was no MitoQ prophylactic effect on exercise-induced lipid peroxidation, lipid soluble antioxidants, and ascorbyl free radical concentration. Although not measured in the current study, it is plausible that MitoQ supplementation did indeed reduce this oxidative stress response; however, non-mitochondrial sources of RONS could misrepresent the results. This notion has been supported by others, whereby discrepancies in circulating oxidative stress biomarkers are not accurately indicative of intracellular (or more specifically, intra-mitochondrial) RONS production (Alleman *et al.*, 2014).

With that being said, this study offers inaugural, mechanistic insights to mitochondrial RONS dynamics as a function of MitoQ supplementation and exercise; however, it is not without limitations, which should be considered for future research. Firstly, given the notion that an exercise intensity and/or duration threshold must be achieved to quantify the deleterious effects of exercise-induced oxidative stress, it would be interesting to quantify intra-exercise blood indices during active recovery to examine the cumulative effects of each work bout. It would also be of interest to

quantify MitoQ concentration in blood to determine whether 20mg of MitoQ is sufficient to induce biological effects, and to support the data observed within this study. Finally, more specific techniques for the quantification of exercise-induced oxidative stress such as confocal/two-photon fluorescence microscopy and Seahorse XFe96 analysis in isolated mitochondria would further clarify the physiological underpinning mechanisms associated with mitohormesis and mitochondrial-targeted antioxidants.

Chapter Seven

Chapter 7:
Synthesis of Findings

The following will determine if the null hypothesis aligned to each experimental study can be rejected or accepted.

7.0 Testing of the Null Hypothesis

Study 1 - *Exogenous Plant-Based Nutraceutical Supplementation and Peripheral Mononuclear Cell DNA Damage Following High Intensity Exercise*

H_0 = Maximal, exhaustive exercise will not induce a state of oxidative stress as confirmed via biomarkers.

Null Hypothesis Rejected

An acute bout (8.7 ± 0.5 mins) of incremental exercise to exhaustion caused significant damage to DNA and lipids as quantified by the comet assay and lipid hydroperoxides respectively. These responses occurred in tandem with the presence of the ascorbyl free radical and activation of circulating superoxide dismutase.

H_0 = A plant-based nutraceutical will not attenuate biomarkers associated with exercise-induced oxidative stress.

Null Hypothesis Accepted

Seven days of dietary supplementation of either a low or high dose of a combination of barley- and wheat-grass juice, failed provide any protective effect against exercise-induced oxidative damage. Although a small increase was detected in circulating gamma-tocopherol and xanthophyll as a result of supplementation, these did not translate into any prophylactic effect. It should be highlighted, that a non-significant protective effect as a result of barley-wheat grass juice supplementation was observed.

Study 2 - *The DNA Damage-Repair Response and Systemic Oxidative Stress as a Function of High-Intensity Hypoxic Exercise*

H_0 = High-intensity exercise will not induce DNA damage following exercise in hypoxia.

Null Hypothesis Rejected

Thirty minutes of high intensity exercise caused a significant increase in oxidative DNA damage as quantified by single-strand damage and base oxidation using the comet assay and FPG incubation respectively. Double-strand DNA damage was also detectable using co-localisation of γH2AX and 53BP1 as a function of exercise. These results coincided with an increase in lipid hydroperoxides, ascorbyl free radical concentration, and a reduction in antioxidant capacity.

H_0 = Exercise-induced DNA damage will not be efficiently repaired in hypoxia.

Null Hypothesis Rejected

For the most part, DNA damage was repaired within 4 hours across the single-strand, double-strand, and base oxidation biomarkers following high intensity exercise in normoxia. This repair response was slightly prolonged in the hypoxic conditions; single-stranded damage, and the γH2AX biomarker returned to baseline within 24-hours, whereas FPG sensitive sites and 53BP1 took 48-hours to exhibit full repair.

Study 3 – *Acute and Chronic Administration of Mitochondrial Targeted Quinone and Mitochondrial DNA Damage following High-Intensity Intermittent Exercise*

H_0 = High-intensity intermittent exercise will not induce lymphocyte and human muscle mitochondrial DNA damage genome.

Null Hypothesis Rejected

Four bouts of high-intensity exercise at 90-95% of maximum heart rate induced significant damage to the mitochondrial genome across both acute and chronic experimental phases. These findings were corroborated in both lymphocyte and human muscle tissue following exercise.

H_0 = Acute and chronic administration of a mitochondrial-targeted antioxidant (MitoQ) will not attenuate mitochondrial DNA damage following exercise.

Null Hypothesis Partially Rejected

An acute 20mg dose of mitochondrial targeted mitoquinone administered 1-hour prior to high intensity intermittent exercise did not provide any prophylaxis against mitochondrial DNA damage or lipid peroxidation. However, twenty-one days of 20mg MitoQ ingestion (per day) protected the mitochondrial genome of both lymphocyte and human.

7.1 Summary of Experimental Research

Work contained within examined the role of high-intensity exercise in DNA damage alongside the quantification of other indices of oxidative stress. Although the underlying theme within the thesis is associated with exercise-induced DNA damage, investigations into the effect of normobaric hypoxia, and antioxidant supplementation were also conducted. In study one, the effects of a novel plant-based nutraceutical on exercise-induced oxidative stress was examined. The second study detailed the DNA damage-repair response following exercise in both hypoxia and normoxia. Finally, the third experimental study determined the efficacy of mitochondrially-targeted antioxidant on markers of DNA damage and oxidative stress. The following section focuses on summarising and synthesising the findings of this body of work.

7.2 Discussion of Findings

7.2.1 Exercise and Oxidative Damage

All experimental studies (Chapters 4, 5, and 6), irrespective of exercise modality, induced oxidative damage to DNA. Following an incremental treadmill test to exhaustion (Chapter 4), DNA damage as assessed by the comet assay, increased by an average (all groups pooled) of Δ13.4% from baseline; it should be noted DNA damage was greater in the control group. Chapter 5 contrasted the effects of normoxic and hypoxic exercise on the DNA damage-repair response. In accordance with the previous study, there was a significant increase in single-strand DNA damage following 30 minutes of exercise at 80-85% of relative $\dot{V}O_{2max}$; this was accompanied with an increase in FPG base oxidation. This was the first study to use dual-staining immunohistochemistry to quantify double-strand DNA damage following exercise. The results of this experiment indicate high-intensity exercise can cause oxidative damage

to multiple markers of DNA. It should be noted, with the exception of base oxidation, all biomarkers were exacerbated within the hypoxic condition. Finally, the concluding experimental study aimed to investigate the effect of high-intensity intermittent exercise. Following 4 bouts of 4 minutes at 90-95% HR_{max}, nuclear DNA single-strand damage increased within lymphocytes. This was accompanied by an increase in mitochondrial DNA damage within extracted PBMC and human muscle tissue. Collectively, this research supports the notion that exercise causes physiological stress, thereby inducing DNA damage within both PBMC and muscle tissue across both nuclear and mitochondrial genomes. These findings are in accordance with others following exhaustive exercise (Moller et al, 2001, Davison *et al.*, 2005, Fogarty *et al.*, 2013a), with Fogarty *et al.*, (2013b) demonstrating an increase in mitochondrial 8-OHdG following 100 isolated and continuous maximal knee extensions. On the contrary, not all research is in accordance with the findings of this thesis (Briviba *et al.*, 2005; Peters *et al.*, 2006; Wagner *et al.*, 2010). For one, Briviba and colleagues (2005), attributed the discrepancy between endurance running and a lack of oxidative DNA damage to a potential protective effect of antioxidants; additionally, due to the recreational nature and self-selected pace of the race, the relative intensity of the exercise was not controlled for. In the case of Peters *et al.* (2005), the sample population was comprised of well-trained endurance athletes (Mean $\dot{V}O_{2max}$; 60.6 ± 1.8) completing a total weekly training volume of 60-90-km. Thus, a probable cause for the non-significant increase in DNA damage could be due to the beneficial adaptations of regular exercise; i.e. reduced basal production of oxidants, increased antioxidant defences, and a reduction in radical leakage during oxidative phosphorylation (Leeuwenburgh & Heinecke, 2001; Simioni *et al.,* 2018). Finally, in the instance of Wagner *et al.* (2010), the sample population was comprised of well-trained Ironman athletes and is therefore subject to the aforementioned points regarding the training status of individuals. In addition, the authors state the purpose of the study was to investigate 'real' race conditions and participants were allows to consume antioxidants *ad libitum*; this was confirmed by an increase in plasma ascorbic acid and α-tocopherol concentrations which may counteract the effect of exercise-induced oxidative stress.

Across all experimental studies (Chapters 4, 5, and 6) high-intensity exercise (maximal vs. constant work rate vs. intermittent) caused oxidative stress, as evidenced by an increase in DNA damage (SSB, base oxidation, DSB) across nuclear and mitochondrial genomes, and changes to other systemic measures, such as: lipid

hydroperoxides, lipid soluble antioxidants, the ascorbyl free radical, and superoxide dismutase activity (Chapter 4 only). Fogarty and colleagues (2011) reported DNA damage following 5 minutes of moderate- and high-intensity exercise, but not at a low intensity. This increase in DNA damage was in conjunction with an increase in lipid hydroperoxides and the presence of alkoxyl radicals. In addition, they conclude this oxidative damage to the membranes of mononuclear cells can expose intracellular DNA, further propagating DNA damage (Quindry *et al.*, 2003). Using DNA damage as a primary outcome measure, across each of the three experimental trials, single-strand DNA damage as a function of exercise (control groups only) increased by 18.2%, 32.7%, 34.12% (acute phase of chapter 6), and 28.9% (chronic phase of chapter 6) respectively. The magnitude and variation of this change in DNA damage observed within chapter 4 is likely to be multifactorial. For one, the exercise modality used in chapter 4 (running) was different than that employed in the rest of the experimental studies (cycle ergometer). Due to the whole-body nature of running, $\dot{V}O_{2max}$ is on average, 5-10% higher on the treadmill than on a cycle. It is generally accepted that in exercise situations involving a greater muscle mass, as in running, the higher the $\dot{V}O_{2max}$ value (Millet *et al.,* 2009). However, although whole-body oxygen uptake may be greater during the incremental running exercise as observed in chapter 4, it would be plausible to hypothesis that the cycling exercise in chapters 5 and 6, required a greater demand for oxygen at the skeletal muscle cellular level; thus, could explain the lower DNA damage observed within the treadmill experimental study (Scott *et al.*, 2006). With that being said, research by Richardson and colleagues (2001) would suggest that once exercise intensity exceeds 50-60% of $\dot{V}O_{2max}$, intracellular partial pressure of oxygen plateaus. This would suggest that neither the exercise intensity, nor the modality of exercise accounts for the variation between DNA damage observed in the first study. However, there does appear to exist an exercise intensity and/or duration threshold which must be achieved in order for oxidative damage to be detectable. For example, Bloomer *et al.* (2007), varies exercise duration between 30-, 60-, and 120-minutes but maintained an exercise intensity of 70% $\dot{V}O_{2max}$. They reported an increase in oxidative stress following 120 minutes of cycling in anaerobically trained men. Indicative of the data, each of the exercise protocols within this thesis achieved the intensity threshold (maximal vs. 80-85% $\dot{V}O_{2max}$ vs. 90-95% HR_{2max}); however, discrepancies between the DNA damage observed in the chapter 4 in contrast with chapters 5 and 6, may be explained by the duration spent at a higher

exercise intensity (total work: chapter 4 – 8.7 minutes [TTE]; chapter 5 – 30 minutes; chapter 6 – 16 minutes). A summary of the experimental studies can be found in Table 7.1.

Table 7.1. A summary of the exercise variables and DNA damage across the 3 experimental studies.

	Experimental Study 1	**Experimental Study 2**	**Experimental Study 3**
DNA Damage (Comet Only)	18.2%	32.7%	*Acute:* 34.12% *Chronic:* 28.9%
Exercise Modality	Treadmill	Cycle Ergometer	Cycle Ergometer
Intensity	Graded Exercise Test	80-85% $\dot{V}O_{2max}$	90-95% HR_{2max}
Total Work	8.7 minutes	30 minutes	16 minutes

It is likely the increase in oxidative damage to DNA and lipids are attributed to an increase in RONS production as a function of exercise. There are a number of mechanisms associated with exercise and free radical production such as the mitochondria, NADPH-oxidases, neutrophil activation, and xanthine oxidase, which could all initiate the accumulation of superoxide; in turn, shifting to a more oxidatively stressed state. In doing so, the antioxidant capacity becomes compromised (as evident in Chapter 5), leading to the generation of a more damaging cell environment; this is in accordance with others (Davison *et al.*, 2006; Davison *et al.*, 2008). It is plausible the nature of exercise causes an increase in liable transition metals and subsequent formation of the undiscriminant hydroxyl radical via Fenton-mediated mechanisms. Due to chemical reactivity, and technological limitations, it is not possible to accurately determine the hydroxyl radical in a biological medium, however, it is highly likely this is the primary radical for initiating damage to biologically important molecules. For example, hydroxyl-mediated hydrogen abstraction in lipids containing carbon double bonds, specifically polyunsaturated fatty acids (due to their activated methylene bridge) results in the generation of lipid-derived peroxyl radicals and hydroperoxides (Yin *et al.*, 2011). Although a number of downstream ramifications can arise from this process, the primary consequence is the structural modifications to membrane permeability and

fluidity; thus, exposing intracellular lymphocyte DNA which may increase the likelihood of secondary lipid radicals damaging purine or pyrimidine bases (Fogarty *et al.*, 2011).

In the second experimental investigation (Chapter 5), this exercise-induced attack on lipids was exacerbated in 12% hypoxia; this response is in accordance with others (Davison *et al.*, 2008; Fogarty *et al.*, 2011), and discordant with similar previous research (Lovlin *et al.*, 1987; Quindry *et al.*, 2013). It is plausible that additional mechanisms within hypoxia compounded the oxidative stress response. For example, high proton motive force, increased ubiquinol/ubiquinone, and increased NADH/NAD^+ ratio activates a superoxide burst following initial exposure to hypoxia; thought to originate from mitochondrial oxidative phosphorylation (Hernansanz-Agustin *et al.*, 2014). The underlying causative factor for this response is through reductive stress, whereby mitochondria become inefficient and a resultant accumulation of reducing equivalents. Secondly, during the initial responses to hypoxia, as arterial saturation of oxygen declines, there is an increased concentration of vascular epinephrine and NO· (Mazzeo *et al.*, 1998); these substrates may be autoxidated propagating superoxide formation, or in the case of NO· reacting with superoxide, a peroxynitrite-mediated generation of hydroxyl radicals may ensue (Yuan *et al.,* 2003). Finally, as proposed by Moller *et al.*, (2001) and confirmed through the work within this study, high-intensity exercise in combination with hypoxia, decreased selective antioxidants, thus potentially leaving DNA and lipids vulnerable to oxidative attack.

Unsurprisingly, the observed trends within the DNA damage data coincided with the lipid peroxidation data outlined throughout this thesis. Initially, it was confirmed that incremental, maximal exercise to exhaustion induced oxidative damage to DNA and lipids; although not measured, it is plausible this is through the exercise-mediated generation of superoxide and other oxygen radicals. Following 30 minutes of exercise at 80-85% $\dot{V}O_{2max}$, there was substantial damage on all indices of DNA damage (i.e. single-strand, double-strand, and base oxidation). Mechanistically, it is generally well accepted that the hydroxyl radical is the primary initiator of DNA damage with addition reactions to the 5,6-pyrimidine and 7,8-purine double bonds of nucleobases and secondary hydrogen abstractions of thymine and 5-methylcytosine being some of the most common (von Sonntag, 1987; Cadet *et al.*, 2010; Wagner & Cadet, 2010). With more than 100 known hydroxyl-mediated lesions, and given the undiscriminant nature of this radical, it is impossible to predict the type of oxidative lesion; thus, DNA damage was investigated across single- and double-stranded biomarkers, in addition to FPG

incubation to quantify base oxidation. The observed increases in single-strand DNA damage and base oxidation has been confirmed across a multitude of exercise durations, intensities, and modalities (Azqueta *et al.*, 2014; Soares *et al.*, 2015; Davison *et al.*, 2008; Fogarty *et al.*, 2011), with others demonstrating conflicting results (Briviba *et al.*, 2005; Reichhold *et al.*, 2008; Neubauer *et al.*, 2008). However, experimental evidence involving exercise and double-strand DNA damage is lacking.

7.2.2 DNA Damage-Repair Response

Regardless of the observed DNA damage across all experimental studies (Chapters 4, 5 and 6), mutagenesis of such DNA damage becomes evident as a consequent of inaccurate and/or inefficient repair; as such, the DNA damage-repair response was investigated in experimental study 2 (Chapter 5). DNA repair of single-strand damage and base oxidation was achieved by the 24-hour and 48-hour time points respectively, following high-intensity hypoxic exercise. Although similar trends were observed in normoxia, FPG sensitive sites were repaired by the 24-hour time point. In relation to double-strand repair, *γ*-H2AX and 53BP1 demonstrated full repair within 2-hours and 4-hours respectively following exercise in normoxia. This contrasts with the hypoxic condition which demonstrated a slightly exacerbated response with *γ*-H2AX and 53BP1 foci achieving full repair within 4-hours and 24-hours respectively. Although this study is one of the first to comprehensively characterise the DNA damage-repair response as a function of exercise, the data obtained is insufficient to determine the mechanisms of repair for each of the measured DNA lesions. With that being said, using sound theoretical underpinning knowledge, it is proposed that a number of pathways are working in collaboration to ensure the rapid, and efficient repair of potentially mutagenic DNA damage.

In relation to double-strand DNA repair, the author suggests that this may be achieved through the non-homologous end joining pathway due to the trends presented by the detection of 53BP1. Furthermore, 53BP1 inhibits the repair protein BRCA1; a key regulator of homologous recombination repair (Bouwman *et al.*, 2010). As a result, the homologous recombination repair pathway is downregulated and 53BP1 promotes the non-homologous end joining repair pathway (Mirza-Aghazadeh-Attari *et al.*, 2019; Gupta *et al.*, 2013). Although these propositions may be somewhat speculative, it is important to note the present data provides a foundation of research for future experimental investigations to be constructed upon.

With respect to repair of single base oxidation damage (as assessed by FPG incubation), the data within Chapter 5 depicts the DNA damage-repair response up to 72-hours following exercise. These small alterations to bases are mainly repaired by base excision repair; briefly, a lesion-specific glycosylase removes the damaged base, and is subsequently filled and sealed by DNA polymerase and ligase, respectively (Evans *et al.,* 2000). The use of the modified comet assay with formamidopyrimidine DNA glycosylase incubation in Chapter 5, allows for the detection of 8-hydrodeoxyguanine and open-ringed purines (Collins, 2002) which converts these damaged bases to breaks; thus, increasing the comet tail intensity as a result of oxidised purines. Collins (2002), also proposes that DNA repair rate generally coincides with the amount of damage; thus, DNA repair will be greatest in the initial stages following oxidative insult. This hypothesis coincides with the data depicted in Figure 5.2B with the greatest rate of repair between the immediately post-, and the two-hour post-exercise time point. It is worth highlighting that the use of the FPG enzyme to detect DNA repair within lymphocytes has been successfully used by others (Humphreys *et al.,* 2007; Cemeli & Anderson, 2011); thus the data in Chapter 5 further supports the validity of such approaches to monitor DNA repair.

7.2.3 Exercise-Induced Oxidative Stress and Supplementation

For the most part, the body of work within this thesis largely focuses on oxidative damage as a result of high-intensity exercise; however, two of the experimental studies (Chapter 4 and 6), were comprised of antioxidant supplementation in an attempt to mitigate some of the potential deleterious effects of exercise-induced DNA damage.

The susceptibility of DNA and lipids to oxidative attack increased as a result of a graded exercise test to exhaustion. A combination of barley- and wheat-grass juice, failed to reduce DNA damage or lipid peroxidation following 7-days of supplementation with a low- or high dose; however, small increases in plasma gamma-tocopherol, xanthophyll, and superoxide dismutase concentration were observed. Yi *et al.* (2011) and Shyam *et al.*, (2007) demonstrated a reduction in biomarkers associated with oxidative stress including, urinary 8-OHdG, MDA, and reduced glutathione following supplementation of wheatgrass for 14 days and 30 days respectively. It is plausible the length of supplementation was insufficient to detect changes in exercise-induced oxidative stress. In support of this, the plant-based nutraceutical resulted in marginal, albeit insignificant changes in DNA damage and lipid peroxidation. With that being

said, Fogarty and colleagues (2012), reported a prophylactic effect on DNA damage and lipid peroxidation following acute and chronic watercress supplementation following exercise. They concluded there was no detectable prophylactic difference between the acute and chronic phases; indicating that supplementing for 8-weeks provided no additional benefit. Despite administrating both low- and high-doses of the supplement, further analysis of the supplement would be required to contrast between the antioxidant profile of these doses.

The findings of the third, and final experimental investigation (Chapter 6) demonstrated a potent protective effect against mitochondrial DNA damage following 21 days of MitoQ supplementation; the antioxidant properties of MitoQ have been corroborated by others (Kelso *et al.*, 2001; Asin-Cayuela *et al.*, 2004; Adlam *et al.*, 2005; James *et al.*, 2007; Neuzil *et al.*, 2007). With that being said, no effect was reported from acute supplementation of 20mg MitoQ 1-hour prior to high intensity intermittent exercise. Although circulating, and indeed mitochondrial concentrations of MitoQ were not measured within this experimental investigation, it is highly plausible that allowing 1-hour between administration, and the onset of exercise was insufficient for MitoQ uptake and accumulation within the mitochondria. This postulation is supported by the observed prophylactic effect on PBMC and muscle mitochondrial DNA damage following 21-days of chronic supplementation.

MitoQ offered no prophylaxis against exercise-induced lipid peroxidation. Lipid hydroperoxides reduced across both acute and chronic phases to similar degrees; although this was not significant. It is plausible the quantification of lipid peroxidation in this study lacked specificity to detect changes in mitochondrial lipid peroxidation, and indeed whether any prophylactic effect was present. Mitochondria are particularly susceptible to lipid peroxidation due to the high abundance of anionic cardiolipin, phosphatidylethanolamine, and phosphatidylcholine within the inner mitochondrial membrane (Schlame & Greenberg, 2017). Oxidation of these lipids has the potential to initiate a number of downstream ramifications including propagating further mitochondrial DNA damage and lipid peroxidation (Wong-ekkabut *et al.*, 2007; Ademowo *et al.*, 2017). The administration of mitochondrial-targeted antioxidants to reduce lipid peroxidation has previously been reported, especially within clinical populations (Gane *et al.*, 2010; Snow *et al.*, 2010; Smith *et al.*, 2011), and supported by others (Ma *et al.*, 2015; Hao *et al.*, 2016; Reddy *et al.*, 2017). With that being said, the present data fails to corroborate these findings. On the contrary, it should not be

excluded that MitoQ did indeed offer a prophylactic effect following high-intensity intermittent exercise. Given the data obtained from the present study, it is impossible to determine if the observed DNA damage (and subsequent reduction following MitoQ) was caused by direct hydroxyl radical attack, or from a combination of peroxidative-driven secondary damage. It is physiologically conceivable that MitoQ administration did reduce mitochondrial lipid peroxidation; however, this is speculative, and future research should incorporate measures of lipid peroxidation specific to isolated mitochondria (Willis, 1966; Kaur *et al.*, 2007; Wani *et al.*, 2011).

7.3 Recommendations for Future Research

Throughout the writing of this thesis a number of areas have been highlighted to the author's curiosity which require further understanding, and thus it would be prudent to recommend several areas for future experimental investigations.

(1) The findings of the second experimental investigation (Chapter 5), although novel and indeed progressive, arguably offers more questions than answers. The study itself characterises the DNA damage-repair response following constant-work, high intensity exercise in hypoxia. Although the data provides a solid foundation for future research, many of the mechanistic conclusions are inferences based on the theoretical underpinning of radiation-induced DNA damage-repair reports. It would be informative for future research to include analysis of gene expression and protein concentration, to not only identify the pathways of DNA repair, but to determine if the corresponding repair pathways coincide with the data observed in the present study. For example, research surrounding exercise-induced double-strand DNA damage is lacking, and more so, data on the requirements for the repair of these lesions. One such avenue, could potentially be the determination of double-strand break repair following high-intensity exercise. Due to the interplay between 53BP1 and BRCA1 proteins, and the non-homologous end joining and homologous recombination repair pathways, the analysis of associated genes and proteins in conjunction with measures of repair to the damaged DNA sequence (Figueroa-Gonzalez & Perez-Plasenci, 2017), would provide further clarification of *in vivo* repair of double-strand damage following exercise.

(2) Keeping in line with the research presented in chapter 5, it is important to consider the environmental conditions and the sample population used within this study. The majority of evidence within the area of exercise and oxidative stress has

been conducted at sea-level and although the oxidative biomarkers fundamental to altitude research are identical to those collected for sea-level exercise, altitude-based research requires consideration of methodological intricacies. For one, examination of redox-sensitive gene transcripts related to mitochondrial biogenesis, endogenous antioxidant up-regulation, and cellular stress responses reflects the acute exercise stimulus and holds important implications for the adaptive response to exercise at altitude (Powers *et al.,* 2011). Secondly, both the hypobaric (Faiss *et al.,* 2013) and hypoxic (Debevec *et al.,* 2014) aspects of high altitude appear to have independent influences on the resultant oxidative stress, though the underlying mechanisms are not resolved. Furthermore, there appears to be subtle, but important differences in lowland dwelling versus native highland people. One notable difference is that high altitude natives appear to have higher blood concentrations of reduced glutathione/oxidized glutathione and SOD as compared with altitude acclimated or unacclimated lowlanders (Sinha *et al.,* 2009). However, firm conclusions about these populations may not be warranted currently as the collective blood antioxidant profile, in particular for low molecular weight fat- and water-soluble antioxidants, was not consistently elevated in highlanders as compared with their lowland counterparts (Sinha *et al.*, 2009). Whether these findings are influenced by diet in addition to genetic and other environmental factors is not currently resolved and merits further investigation before establishing firm conclusions on the matter. Finally, lowland natives who are acclimated to altitude appear to have a modulated oxidative stress response in both unstressed resting and post-exercise scenarios, though these outcomes are less robust than in highland natives. Understanding of the independent and combined influence of hypoxic and oxidative stress stimulus of exercise at high altitude is still in the formative stages. This conclusion is perhaps most notable for the adaptive responses induced during exercise and the exercise recovery phase. Future research should be conducted in reductionist investigations of oxidative stress in order to understand the cellular level responses that underpin physiological outcomes to exercise at high Altitude. Furthermore, examinations between lowlanders versus altitude acclimated lowlanders versus native highlanders warrants research, with special consideration given to the role of exercise and DNA damage.

(3) The full potential of EPR was underappreciated in the context of experimental study two (Chapter 5); although the ascorbyl free radical was assessed, quantifiable conclusions are limited. For example, determining the exact mechanism

for the generation of the ascorbyl radical within this study was tangential, however it is possible that several radicals, including lipid peroxyl and alkoxyl radicals were also involved. To further elucidate the mechanisms associated with free radical generation and DNA damage within a hypoxic environment, future research using EPR to investigate lipid peroxyl, alkoxyl, and ubisemiquinone radicals would be invaluable.

(4) It is also worth noting, that the reported data for the DNA damage-repair response for the 24-, 48, and 72-hour time points was obtained from the immediately post-exercise time points and allowed to culture *in vitro*. Although the findings coincide with the literature, it cannot be disregarded that extracting samples at the above time points would provide a more accurate representation of the DNA damage-repair response *in vivo*. Furthermore, contrasting the DNA damage-repair response between cultured *in vitro* samples, and newly extracted *ex vivo* samples at these time points would provide a measure of reliability and continuity between sample types.

(5) Finally, the concluding experimental investigation (Chapter 6) offered fundamental insight to mitochondrial DNA damage and the potential for mitochondrial targeted antioxidant supplementation as a function of high-intensity intermittent exercise. However, upon development of the chapter, several interesting questions arose. Firstly, from a basic research design, and sampling point of view, it is evident that an intensity/duration threshold exists whereby oxidative stress in manageable until exercise is physiologically challenging to induce oxidative damage. Thus, it would be interesting to investigate the effect of each 4-minute bout of high-intensity exercise to determine the cumulative effect of each bout. Secondly, determining the physiological reason as to why no prophylactic effect was observed in the acute phase is limited and also, speculative. Although muscle samples were not taken in the acute phase, the addition of muscle biopsies, and quantifying the concentration of MitoQ (Rodriguez-Cuenca *et al.*, 2010) within all tissue types and during both phases would offer a clearer mechanistic explanation for the observed findings. Furthermore, the addition of fluorescence probes (such as MitoSOX-red, CM-H_2DCFDA, dihydrorhodamine-123, MitoPY1, MitoDEPMPO and C11-BODIPY$^{581/591}$) in tandem with epifluorescence and/or confocal laser scanning microscopy would offer greater sensitivity to the mechanisms at work associated with exercise-induced damage to mitochondrial DNA (Forkink *et al.*, 2010). Fourthly, exercise-induced lipid peroxidation alters the inner cell membrane fluidity and functionality, and modifies the protein complexes of the electron transport chain (Wong-ekkabut *et al.*, 2007; Ademowo *et al.*, 2017); as such, it would

be prudent to employ the Seahorse XFe96 assay in isolated mitochondria to assess membrane and ETC complex functionality following exercise-induced damage, and to determine if MitoQ has pro-oxidant properties. As a final point, mitochondrial RONS-mediated signalling, downstream targets, and the possible positive/negative effects of MitoQ supplementation have been discussed and proposed in Chapter 6. Given the current nature and direction of exercise redox biology, it is recommended that future research focuses on *downstream* ramifications of mitochondrial oxidative damage, and the potential implications that targeted supplementation may have on these processes.

7.4 Limitations of this Work

Limitations of the work contained in this thesis should be considered when interpreting the data, and these should inform future research with a similar methodological design.

There are a number of limitations associated with chapter 4, which if addressed, could provide further clarity to the changes in biomarkers of oxidative stress as a function of barley-wheat grass juice supplementation. For one, analysis of the composition of the product was not performed prior to the study design. Previous research on barley- and wheat-grass provided the basis for selection of these plants due to their high antioxidant profiles. Performing UHPLC-MS on the specific batch of products, would provide clarity on dosing protocols for future research using similar methodological designs. For this reason, a low- and high-dosing strategy was used to allow for detection (if any), in dose-dependent changes in biomarkers of exercise-induced oxidative stress. Finally, although the study incorporated a three-arm crossover design, it is plausible that by increasing the sample size a significant statistical change may be detectable.

The main limitation in Chapter 5 involves the limited data regarding the mechanistic nature of DNA repair, especially in the context of double-strand repair. Although this study clearly identifies an increase in DNA damage following high-intensity exercise, and a subsequent reduction (indicative of DNA repair), the data doesn't allow for identification of repair pathways. Lastly, although the data obtained from the 24-, 48-, and 72-hour time points coincide with the literature regarding DNA repair, no comparison was made between the *in vitro* samples, and freshly extracted *in vivo* samples acquired at these time points.

Chapter 6 robustly determines mitochondrial DNA damage in PBMC and human muscle through the use of long-amplicon PCR; in addition to nuclear DNA damage

from PBMC. However, during the acute supplementation phase, blood concentration of MitoQ was not quantified; this would provide insight as to whether the 1-hour absorption phase is long enough to cause a physiological effect. Although the aims of the study were achieved, additional factors may have provided greater mechanistic clarity; for one, measurement of mitochondrial membrane potential, electron transport chain efficiency, and mitochondrial lipid peroxidation. Finally, a clear reduction in mitochondrial DNA damage was reported following chronic MitoQ supplementation; however, it is unknown if this has downstream ramifications on mitochondrial signalling pathways associated.

Finally, there are a number of methodological considerations which collectively apply across all experimental studies throughout this thesis and interpretation of the findings must be done so with caution to prevent generalisations across to other sample populations:

1. *Training Status of Participants*; The exercise-induced increase in RONS have important implications in oxidative damage, as well as in the functional performance of the organs. However, it is also becoming clear that individuals engaged in chronic exercise are more resistant to oxidative stress, mainly due to the adaptation of their antioxidant defence systems. Regular/moderate exercise has been shown to enhance antioxidant defense by incrementing the activity of endogenous antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, and catalase (Miyata *et al.*, 2008). Exercise protects the body against constant mild or moderate ROS exposure through redox-associated preconditioning including oxidative damage repair systems (Radak *et al.,* 2013). This moderate exercise-mediated adaptation also involves increased myocellular antioxidant capacity which helps to lower RONS levels. Caution is warranted in the design of future research if populations include elite/high-trained athletes, or physically inactive/sedentary populations as this could under- or over-estimate the outcomes.
2. *Sex:* The presented research was all conducted on a male population due to restrictions associated with time, logistics, and resources; however, the author highlights the need for male versus female research to ascertain if physiological and redox regulatory differences exists throughout the female menstrual cycle. Research by Serviddio and colleagues (2002) have postulated that the peak oxidative stress phase occurs in the central phases of the cycle (late follicular phase and early luteal phase), or rather at the time of ovular maturation and

possible implantation. This phase occurs with: a) an increase in the production of GPx; b) a reduction in the GSH; c) an increase in the GSSG; d) a substantial stability of the MDA. Additionally, Cornelli *et al.* (2013), concluded that healthy eumenorrheic women go into a state of oxidative stress for two-thirds of the menstrual cycle. Currently it is unknown what this means in the context of exercise; however, the author suggests that menstrual variables and sampling time points need to be accounted for in female-only trials, and if sample populations contain a combination of males and female, that the findings need to be interpreted with caution.

3. *Age:* Among the theories that explain the aging process, the free radical theory of aging is long-established (Harman, 1956). This theory speculates that aging is a consequence of the failure of several defensive mechanisms to respond to the RONS-induced damage, particularly at the mitochondria (Islam, 2017). Age-related diseases are related to structural changes in mitochondria, accompanied by the alterations of biophysical properties of the membrane including alteration in the electron transport chain complexes activities, decreased fluidity, and subsequently resulted in energy imbalance and mitochondrial failure. These perturbations impair cellular homeostasis and mitochondrial function and enhance vulnerability to oxidative stress (Eckmann *et al.,* 2013; Chistiakov *et al.,* 2014). Elderly people are susceptible to oxidative stress due to a decline in the efficiency of their endogenous antioxidant systems. The mean age of the participants across each of the experimental studies were 21.5 ± 2, 22 ± 2, and 25 ± 4 respectively; thus, care should be taken when translating these findings to elderly populations.

7.5 Concluding Remarks

The work in this thesis has enhanced the knowledge and understanding of exercise-induced DNA damage and oxidative stress with the following novel findings;

- High-intensity exercise (maximal vs. 80-85% $\dot{V}O_{2max}$ vs. 90-95% HR_{max}) causes oxidative damage to DNA and lipids; however it appears duration spent at these intensities (chapter 4 – 8.7 minutes [TTE]; chapter 5 – 30 minutes; chapter 6 – 16 minutes) has a greater effect on the oxidative damage responses.
- There is potential for the use of natural plant-based products to reduce exercise-induced DNA damage and lipid peroxidation. This could be useful for mitigating the dampened response to exercise adaptive signalling following synthetic antioxidant supplementation.
- A single bout of high-intensity, steady-state exercise induces double-strand DNA damage which is exacerbated by hypoxia.
- High intensity intermittent exercise induces oxidative damage to the nuclear and mitochondrial genomes. The oxidative insult to mitochondrial DNA was attenuated by chronic supplementation of MitoQ, but not when administered 1-hour prior to exercise.

Chapter Eight

Chapter 8:
Bibliography

8.0 Cited Literature

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Appendix A

FC39

UNIVERSITY OF ULSTER RESEARCH GOVERNANCE

RG3 Filter Committee Report Form

Project Title	The Effects of Aerobic Exercise and Barley Grass Supplementation on DNA Damage
Chief Investigator	Prof G Davison
Filter Committee	School of Sport

This form should be completed by Filter Committees for all research project applications in categories A to D (*for categories A, B, and D the University's own application form – RG1a and RG1b – will have been submitted; for category C, the national, or ORECNI, application form will have been submitted)

Where substantial changes are required the Filter Committee should return an application to the Chief Investigator for clarification/amendment; the Filter Committee can reject an application if it is thought to be unethical, inappropriate, incomplete or not valid/viable.

Only when satisfied that its requirements have been met in full and any amendments are complete, the Filter Committee should make one of the following recommendations:

The research proposal is complete, of an appropriate standard and is in

- category A and the study may proceed* ☐
- category B and the study must be submitted to the University's Research Ethics Committee** Please indicate briefly the reason(s) for this categorisation ☐

  [ ]
- category C and the study must be submitted to ORECNI along with the necessary supporting materials from the Research Governance Section*** ☐
- category D and the study must be submitted to the University's Research Ethics Committee** ☒

Signed: [signature] Date: 11/6/15

Chairperson/Administrator of Filter Committee

*The application form and this assessment should now be returned to the Chief Investigator. The Filter Committee should retain a copy of the complete set of forms.

** The application form and this assessment should now be returned to the Chief Investigator so that he/she can submit the application to the UUREC via the Research Governance section. The Filter Committee should retain a copy of the complete set of forms for their own records.

*** The application form and this assessment should now be returned to the Chief Investigator so that he/she can prepare for application to a NRES/ORECNI committee. The Filter Committee should retain a copy of the complete set of forms for their own records.

For all categories, details of the application and review outcome should be minuted using the agreed format and forwarded to the Research Governance section

FC 35

UNIVERSITY OF ULSTER RESEARCH GOVERNANCE

RG3 Filter Committee Report Form

Project Title	Effects of exercise in hypoxia on DNA oxidation, repair and gene expression
Chief Investigator	Prof Gareth Davison
Filter Committee	School of Sport

This form should be completed by Filter Committees for all research project applications in categories A to D (*for categories A, B, and D the University's own application form – RG1a and RG1b - will have been submitted; for category C, the national or ORECNI, application form will have been submitted).

Where substantial changes are required the Filter Committee should return an application to the Chief Investigator for clarification/amendment; the Filter Committee can reject an application if it is thought to be unethical, inappropriate, incomplete or not valid/viable.

Only when satisfied that its requirements have been met in full and any amendments are complete, the Filter Committee should make one of the following recommendations:

The research proposal is complete, of an appropriate standard and is in

- category A and the study may proceed* ☐
- category B and the study must be submitted to the University's Research Ethics Committee** Please indicate briefly the reason(s) for this categorisation ☐

- category C and the study must be submitted to ORECNI along with the necessary supporting materials from the Research Governance Section*** ☐
- category D and the study must be submitted to the University's Research Ethics Committee** ☒

Signed: [signature] Date: 15/6/15

Chairperson/Administrator of Filter Committee

*The application form and this assessment should now be returned to the Chief Investigator. The Filter Committee should retain a copy of the complete set of forms.

** The application form and this assessment should now be returned to the Chief Investigator so that he/she can submit the application to the ULREC via the Research Governance section. The Filter Committee should retain a copy of the complete set of forms for their own records.

*** The application form and this assessment should now be returned to the Chief Investigator so that he/she can prepare for application to a NRES/ORECNI committee. The Filter Committee should retain a copy of the complete set of forms for their own records.

For all categories, details of the application and review outcome should be minuted using the agreed format and forwarded to the Research Governance section.

Version 3 (10/07)

FC 66 2017-18

UNIVERSITY OF ULSTER **RESEARCH GOVERNANCE**

RG3 Filter Committee Report Form

Project Title	High Intensity Intermittent Exercise and Mitochondrial Targeted Antioxidant Supplementation: Effect on DNA and RNA Modification
Chief Investigator	Prof G Davison
Filter Committee	School of Sport

This form should be completed by Filter Committees for all research project applications in categories A to D (*for categories A, B, and D the University's own application form – RG1a and RG1b – will have been submitted; for category C, the national, or ORECNI, application form will have been submitted).

Where substantial changes are required the Filter Committee should return an application to the Chief Investigator for clarification/amendment; the Filter Committee can reject an application if it is thought to be unethical, inappropriate, incomplete or not valid/viable.

Only when satisfied that its requirements have been met in full and any amendments are complete, the Filter Committee should make one of the following recommendations:

The research proposal is complete, of an appropriate standard and is in

- category A and the study may proceed* ☐
- category B and the study must be submitted to the University's Research Ethics Committee** Please indicate briefly the reason(s) for this categorisation ☐

  ☐
- category C and the study must be submitted to ORECNI along with the necessary supporting materials from the Research Governance Section*** ☐
- category D and the study must be submitted to the University's Research Ethics Committee** ☒

Signed: L. Strapp. Date: 21/2/18

Chairperson/Administrator of Filter Committee

*The application form and this assessment should now be returned to the Chief Investigator. The Filter Committee should retain a copy of the complete set of forms.

** The application form and this assessment should now be returned to the Chief Investigator so that he/she can submit the application to the UUREC via the Research Governance section. The Filter Committee should retain a copy of the complete set of forms for their own records.

*** The application form and this assessment should now be returned to the Chief Investigator so that he/she can prepare for application to a NRES/ORECNI committee. The Filter Committee should retain a copy of the complete set of forms for their own records.

For all categories, details of the application and review outcome should be minuted using the agreed format and forwarded to the Research Governance section

Appendix B

Sport and Exercise Science Research Institute
Faculty of Life and Health Science

School of Sport

Recruitment Email

Participants required for a PhD research project with the Sport and Exercise Sciences Research Institute.

Study Title: *The Effects of Barley Grass Supplementation on Exercise-Induced DNA Damage*

Eligibility:

- Male adults aged 18-30 years
- Recreationally fit (aerobic training 3-5 times per week e.g spinning, circuits, running, sports i.e. football, Gaelic etc)
- Non-smoker
- No family history of cardiopulmonary disorders including respiratory illness, asthma, diabetes, epilepsy, psychiatric illness or blood-borne infectious diseases or an abnormal echo cardiograph trace.
- Potential participants will need to be free from antioxidant/multivitamin supplementation for 4 weeks prior testing.

Participants will be reimbursed to the value of £50 for completion of all trials.

Further Information: Please contact Josh Williamson: Williamson-J5@email.ulster.ac.uk or, Gareth Davison: gw.davison@ulster.ac.uk , 028 90366664 for an information sheet.

Approved by the ethics filter committee of the Sport and Exercise Sciences Research Institute and Ulster University Research Ethics Committe

Sport and Exercise Science Research Institute

Faculty of Life and Health Science

School of Sports Studies

Recruitment Email

Participants required for a PhD research project with the Sport and Exercise Sciences Research Institute.

Study Title: Effects of exercise in hypoxia on DNA oxidation, repair and gene expression

Aim: As part of a PhD we are interested in investigating and understanding the mechanisms as to how exercise damages our DNA, and how our bodies respond to damage.

Eligibility:

Male participants only.

Adults aged 18-30 years

Recreationally fit (Train 3-5 times per week)

Non-smoker

Potential participants will need to be free from antioxidant/multivitamin supplementation for 4 weeks prior testing.

What will be required?

If you decide to volunteer (and meet the eligibility criteria) we will ask you to complete an informed consent and a medical questionnaire. Testing will be divided into 2 stages which are briefly outlined below.

Stage One – Familiarisation: During this stage, you will complete two tests of maximal aerobic capacity (VO2max) from a progressive incremental exercise test on a stationary bicycle to exhaustion, with 1 week between tests to establish the level of exertion during the testing stage. A blood sample of 22ml will be drawn from the forearm vein to provide baseline values.

Stage Two – Testing: You will be required to exercise for 1 hour at 75% on a stationary bicycle in both conditions with a duration of 1 week (maximum of 10 days) between tests. A venous catheter will be inserted into a forearm or hand vein to reduce any discomfort associated with frequent blood draws. Venous blood samples will be taken pre-, immediately post-, 2 hours, 3 hours and 4 hours after exercise.

Further Information: Please contact Josh Williamson: Williamson-J5@email.ulster.ac.uk or, Gareth Davison: gw.davison@ulster.ac.uk, 028 90366664 for an information sheet.

Approved by the ethics filter committee of the Sport and Exercise Sciences Research Institute and Ulster University Research Ethics Committee (UREC).

Sport and Exercise Science Research Institute
Faculty of Life and Health Science

School of Sport

Recruitment Email

Participants required for a PhD research project with the Sport and Exercise Sciences Research Institute.

Study Title: *High Intensity Intermittent Exercise and Mitochondrial Targeted Antioxidant Supplementation; Effect on DNA and RNA Modification*

Aim: As part of a PhD we are interested in investigating and understanding the mechanisms as to how exercise affects our genetic material (DNA), and how our bodies respond to any changes.

Eligibility:

White Caucasian male adults aged 18-30 years

Recreationally fit (Train 3-5 times per week)

Non-smoker

Potential participants will need to be free from antioxidant/multivitamin supplementation for 4 weeks prior testing.

What will be required?

If you decide to volunteer (and meet the eligibility criteria) we will ask you to complete an informed consent and a medical history questionnaire. Testing will be divided into 3 stages which are briefly outlined below.

Baseline – You will be required to complete a baseline VO_{2max} test at the Human Performance Lab at Ulster University. On this visit you will also have data such as height, weight, age etc recorded. This will be carried out approximately 1 week prior to the experimental trails.

Stage One – Acute: During this stage, you will consume a supplement 1 hour prior to a high intensity exercise protocol. Your will be required to complete 4 x 4-minute bouts at 90-95% of maximum heart rate with a 3-minute active recovery between high intensity bouts.

Stage Two – Chronic: You will be required to consume the supplement for 21 days while following normal dietary habits. Following the supplementation period, you will be required to complete the high intensity exercise trial for a second time.

Tissue Samples - You will be required to provide venous blood samples pre- and post-exercise for both phases. Muscle biopsies will only be taken at baseline and pre- and post-exercise of the chronic phase.

Further Information: Please contact Josh Williamson: Williamson-J5@ulster.ac.uk or, Gareth Davison: gw.davison@ulster.ac.uk 028 90366664 for an information sheet.

Approved by the ethics filter committee of the Sport and Exercise Sciences Research Institute and Ulster University Research Ethics Committee (UREC).

Appendix C

Sport and Exercise Science Research Institute

Faculty of Life and Health Science
School of Sport

Participant Information Sheet

The Effects of Aerobic Exercise and Barley Grass Supplementation on DNA Damage

You are invited to take part in a research study as part of Doctoral Research Project. Before you decide whether or not to take part, it is important that you understand the purpose of the research and what you will be asked to do. Make sure that you are happy before you decide what to do. Thank you for taking the time to consider this invitation.

1. Background and Aims of the Research

Throughout day-to-day life, our DNA becomes damaged through a number of sources such as oxygen, smoking and pollution. It is thought that unrepaired DNA damage can contribute to the progression of some cancers, particularly colon, breast and prostate cancers.

Studies have concluded that dietary consumption of whole-grains have many health benefits such as protection against cancer, cardiovascular disease and obesity. In relation to the present study, barley grass contains one of the richest sources of antioxidants which is thought to protect against DNA damage.

The primary aim of this study will determine the effects of aerobic exercise and barley grass supplementation on DNA damage. The secondary aims of this study are to examine the effects of barley grass supplementation on red blood cell number.

2. Procedures

Participants will be required to complete an overnight fast (12 hours) prior to testing. Participants will be asked to wear appropriate footwear and clothing for exercising. This may include running trainers, a loose t-shirt/vest and a pair of comfortable shorts.

Stage One – Pre-Experimental Requirements: Following a standard overnight fast (12 hours), you will attend the Ulster University Human Performance Lab exactly two weeks prior to experimental testing to be familiarised with the study protocol and to provide informed consent; this will also include a medical history questionnaire. Standard measurements (age, height, weight) and baseline resting measurements. A total of 20ml of blood will be taken from a prominent forearm vein; this will be performed by an appropriately trained and experienced.
Participants will also be required to complete a familiarisation phase on the treadmill for approximately 10 minutes at low-moderate intensity.

Stage Two – Experimental Testing:

Supplementation with barley grass juice

Participants will be split into one of three groups; they will be comprised of 2 forms of barley grass supplement and a placebo supplement. The supplemental period will last for 14 days followed by a 14 washout period. Normal exercise and dietary habits are to be followed. It should be noted that each group will receive each form of supplement and thus the whole trial will last approximately 3 months.

Exercise protocol

A 12 hour standard overnight fast will be instructed prior to experimental testing; however, subjects can drink water only, and as required. Following measurements of height, weight, age etc., all subjects will complete a maximal exercise test on a treadmill.

Exercise Haematology
Venous blood samples will be taken immediately pre and immediately post experimental testing. A sample of 20ml of blood will be taken pre-exercise and post-exercise respectively. This will be repeated for each of the supplemental groups. 140ml of blood will be taken over the entire study.

3. Risks & Benefits to Participants

There are a number of risks and/or hazards that could potentially occur during testing. These include muscular injury, cardiovascular complications, allergic reaction to barley grass, fainting, bacterial/viral infection, blood borne diseases and bruising from blood samples. Understand that although barley grass is an infrequently consumed food product, an allergic reaction is unlikely. Further, it is possible the barley grass juice may contain gluten and participants with coeliac disease will not be permitted to participate in the study. In the event of severe symptoms and GI disturbances, discontinue use immediately. Contact emergency services or your GP in line with the severity. It should be understood by the participant that the university and research team have taken the necessary actions to minimise the probability of these risks occurring. However, it should be noted that nausea/vomiting and discomfort from blood samples may occur. Maximal exercise testing will be uncomfortable and can produce muscle soreness. There will always be one member of the research team present who is qualified in providing first aid.

Note; if there are any changes in circumstances and/or medical status between completing the medical risk form and the beginning of the study that you let a member of the research team know immediately.

You will be reimbursed the value of £50 for completing the entire study.

4. Issues During The Study
In the very unlikely case that something goes wrong during the study it is important to understand that the university has procedures in place for reporting, investigating, recording and handling adverse events. Any complaints will be taken seriously and should be made to the appropriate authority. This might be the Chief Investigator and/or the University, depending upon who is involved in the research. The University will provide an indemnity statement for research that has been approved through the appropriate governance and ethical review processes.

5. Disclaimer
All data will be held securely and in confidence and that any identifiers will be removed prior to publication as required under Data Protection (1998) legislation.

Participants will be given a randomised subject number in order to keep anonymity so data cannot be traced or used to identify the participant. Data will to be stored on file for 10 years in accordance with University Policy. This means all data will be put into a secure, password-locked electronic file and stored within the University of Ulster. Only the chief investigator, Professor Gareth Davison and I will have access to this data, before being adequately discarded. If someone was to gain access to the data, all information regarding participant identity will remain anonymous.

It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep. You will also be asked to sign a consent form. If you choose to take part, you can change your mind at any time and withdraw from the study without giving a reason.

6. Contact Details
If you have any further questions or queries feel free to contact me on the following information;
Email – Williamson-J5@email.ulster.ac.uk Chief Investigator – gw.davison@ulster.ac.uk

Highlighted practical aspects of the study methodology

You are required to adhere to the following:

- Do not participate in exercise for 48 hrs before coming to the laboratory
- Do not take alcohol before coming to the laboratory
- Following an overnight fast, attend the laboratory for initial screening
- Complete a treadmill run for approximately 10 mins
- Return to the laboratory again, where you will be given a supplement
- Ingest the supplement for 14 days followed by a 14 day washout period
- This sequence will be completed again (i.e supplement for another 14 days)
- While in the laboratory, and for all experimental testing, you are required to exercise on a treadmill
- You are required to provide a blood sample before and after exercise

Sport and Exercise Science Research Institute
Faculty of Life and Health Science
School of Sports Studies

Participant Information Sheet

Effects of Exercise in Hypoxia on DNA Oxidation, Repair and Gene Expression

You are being invited to take part in a research study as part of Doctoral Research. Before you decide whether or not to take part, it is important that you understand the purpose of the research and what you will be asked to do. Please read the following information and do not hesitate to ask any questions about anything that might not be clear to you. Make sure that you are happy before you decide what to do. Thank you for taking the time to consider this invitation.

1. Background and Aims of the Research

It is common knowledge that exercise is good for overall health and well being with a number of benefits, including the decreased risk of cardiovascular disease and stroke. Throughout day-to-day life, our DNA becomes damaged through a number of unavoidable sources; therefore DNA repair is essential for life. This damage comes from a collective of molecules known as reactive oxygen species (ROS). Although these have a number of important functions, they are the primary source of DNA damage within the body in the hours after exercise. It is thought a lack of oxygen leads to extra stress on the body; consequently a greater amount of damage may occur. If damaged DNA is left unrepaired it could contribute to aging and the formation of some cancers.

This project aims to further advance the field of exercise-induced DNA damage and repair. The specific aims of this study are to (a) investigate the means associated with hypoxic exercise-induced damage to DNA, (b) understand the role and regulation of specific DNA repair processes, (c) understand the interaction and mechanisms that antioxidants have on DNA damage and repair, and finally (d) understand how the previous aims adapt in the hours after DNA damage has occurred.

2. Procedures

Participants will be required to complete an overnight fast (8 hours) prior to testing. Participants will be asked to wear appropriate footwear and clothing for exercising. This may include running trainers, a loose t-shirt/vest and a pair of comfortable shorts.

Stage One – Familiarisation: You will attend the Ulster University Human Performance Lab exactly two weeks prior to testing to be familiarised with the study protocol and to provide informed consent; this will also include a medical history questionnaire. At this stage height and weight will be recorded;

heart rate (HR) will also be recorded following a 15 minute rest. A blood sample of 22ml will be drawn from the forearm vein to provide baseline values of biomarkers and oxidative stress; this will be performed by a fully trained phlebotomist.

During this stage, you will complete two tests of maximal aerobic capacity (VO_{2max}) from a progressive incremental exercise test to exhaustion; this will be done in normoxic and hypoxic conditions with 1 week between tests to establish the level of exertion during the testing stage. You will be required to cycle at a cadence of 60rpm on a friction-braked cycle ergometer to produce a power output of 100w. The workload will be increased by 50w every 3min until you can no longer maintain the required work rate. The following criteria will be used to define an achievement of VO_{2max}: attainment of maximum HR (max HR=220-age), a clear peak or plateau in VO_2 values and an RER of >1.0. During the test oxygen uptake will be continuously monitored using an online gas analysis system (Cosmed b2 breath by breath gas analyser) and HR measured.

Stage Two – Testing: You will be required to complete the following protocol in both conditions with a standard duration of 1 week (maximum of 10 days) between tests. A venous catheter will be inserted into a forearm or hand vein to reduce any discomfort associated with frequent blood draws. The catheters will be inserted by an appropriately trained and experienced individual.

Participants will be randomly divided into two groups; one group exercising in normoxia (20.93% inspired of oxygen) and the other in hypoxia, (12% inspired fraction of oxygen). You will exercise for 1 hour at 75% on a cycle ergometer with venous blood samples being taken pre- (22ml) and immediately post- (42ml) exercise.

You will then be asked to come back into the lab exactly 2 hours, 3 hours and 4 hours after the exercise to provide an 8ml blood sample at each time point in order to investigate biomarkers for DNA damage and repair activity.

3. Risks & Benefits to Participants

With all scientific study there are a number of risks and/or hazards that could potentially occur during testing. These include muscular injury, cardiovascular complications, nausea, fainting, bacterial/viral infection, blood borne diseases and bruising from blood samples. It should be understood by the participant that the university and research team have taken the necessary actions to minimise the probability of these risks occurring. However, it should be noted that nausea/vomiting and discomfort from blood samples may occur. It is important that if you experience any of these that you let a member of the research team know as soon as possible. There will always be one member of the research team present who is qualified in providing first aid; Dr John Brown is the Health and Safety Co-ordinator. You will be asked to participate in a physical activity readiness questionnaire which will assess your medical history. This information will be kept confidential and will only be used to assess whether you can participate in the study.
Note; if there are any changes in circumstances and/or medical status between completing the medical risk form and the beginning of the study that you let a member of the research team know immediately.

4. Issues During The Study

In the very unlikely case that something goes wrong during the study it is important to understand that the university has procedures in place for reporting, investigating, recording and handling adverse events. Any complaints will be taken seriously and should be made to the appropriate authority. This might be the Chief Investigator and/or the University, depending upon who is involved in the research. The University will provide an indemnity statement for research that has been approved through the appropriate governance and ethical review processes.

The study procedures and protocols have been reviewed by other people who are knowledgeable in this subject area and by an ethics committee in accordance with university guidelines. If you have any further queries on this do not hesitate to contact the University Research Governance.

5. Disclaimer

All data will be held securely and in confidence and that any identifiers will be removed prior to publication as required under Data Protection (1998) legislation.

Participants will be given a randomised subject number in order to keep anonymity so data cannot be traced or used to identify the participant. Data will to be stored on file for 10 years in accordance with University Policy. This means all data will be put into a secure, password-locked electronic file and stored within the University of Ulster. Only the chief investigator, Professor Gareth Davison and I will have access to this data, before being adequately discarded. If someone was to gain access to the data, all information regarding participant identity will remain anonymous.

It should be noted that data and conclusions from this study will be published in the final PhD thesis; there is also a potential for publication in a scientific journal. It should be reinforced that all identifiers will be removed and identities will be kept anonymous.

It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep. You will also be asked to sign a consent form. If you choose to take part, you can change your mind at any time and withdraw from the study without giving a reason.

6. Contact Details

If you have any further questions or queries feel free to contact me on the following information;
Email – Williamson-J5@email.ulster.ac.uk

Sport and Exercise Science Research Institute
Faculty of Life and Health Science
School of Sport

Participant Information Sheet

High Intensity Intermittent Exercise and Mitochondrial Targeted Antioxidant Supplementation; Effect on DNA and RNA Modification

You are invited to take part in a research study as part of Doctoral Research. Before you decide whether or not to take part, it is important that you understand the purpose of the research and what you will be asked to do. Please read the following information and do not hesitate to ask any questions that might not be clear to you. Make sure that you are happy before you decide what to do. Thank you for taking the time to consider this invitation.

1. Background and Aims of the Research

It is common knowledge that exercise is good for overall health and well-being with a number of benefits, including the decreased risk of cardiovascular disease and stroke. Throughout day-to-day life, our genetic material (DNA/RNA) becomes damaged through a number of unavoidable sources. Our DNA carriers specific information which makes us unique, whereas our RNA uses the information within DNA to create proteins for specific purposes and functions within the body; therefore, repair of our genetic material is essential for life. This damage comes from a collective of molecules known as reactive oxygen species (ROS) which can lead to mutations within DNA/RNA and can contribute to the progression of a number of diseases, such as type II diabetes and cancer. By supplementing with the antioxidant, MitoQ, it is hoped we can minimise this damage. The flow of our genetic material is depicted as;

The aims of this study are to (a) investigate the role of high-intensity intermittent exercise on the damage and repair of genetic material, (b) understand the mechanisms of acute MitoQ supplementation on biomarkers associated with DNA/RNA damage, (c) understand the mechanisms of chronic MitoQ supplementation on biomarkers associated with oxidative damage to DNA and RNA, (d) investigate the regulation of DNA repair as a function of MitoQ supplementation and high intensity intermittent exercise.

2. What do I have to do?

If you choose to take part, you must first be free of antioxidant/multivitamin supplementation for 4 weeks prior to starting the study. It should be noted all participants must be healthy male white Caucasians who are recreationally trained. Participants will be required to complete an overnight fast (8 hours) prior to completing a maximal test to volitional fatigue to provide baseline measurements, which all subsequent exercise trials will be based on. This will be carried out approximately 1 week prior to Stage One. Anthropometric measures such as height, weight and age will also be taken at this point, in addition to venous blood and muscle biopsy samples.

Stage One – Acute Supplementation Phase. Participants will be asked to consume a supplement 60 minutes prior to completing a high intensity exercise trial. The exercise will consist of 4 x 4-minute bouts at 90-95% of maximum heart rate with 3 minutes of active recovery at 70% of maximum heart rate. Blood samples will be taken immediately pre- and post-exercise by a fully trained phlebotomist. Stage One should take no longer than 2 hours to complete.

Stage Two – Chronic Supplementation Phase. Following the acute phase, participants will be asked to consume the assigned supplement for 21 days split between a morning and evening dose. Upon completion of the supplementation period, participants will repeat the exercise trial. Blood and muscle samples will be taken pre- and post-exercise by fully trained individuals. Stage Two experimental testing should be completed within an hour.

A flowchart has been attached to provide an overview as to where blood and muscle samples will take place. Note A total volume of 36ml of whole blood will be taken from each time point.

3. Risks & Benefits to Participants

There are a number of risks and/or hazards that could potentially occur during testing. These include muscular injury, cardiovascular complications, nausea, fainting, and bacterial/viral infection/blood borne diseases and bruising from blood samples. It should be understood by the participant, that the university and research team have taken the necessary actions to minimise the probability of these risks occurring. Furthermore, the potential risks outlined are minimal. However, it should be noted that nausea/vomiting, and discomfort from blood samples may occur. There will always be one member of the research team present who is qualified in providing first aid.

Note; if there are any changes in circumstances and/or medical status between completing the medical risk form and the beginning of the study that you let a member of the research team know immediately.

4. What if Something Goes Wrong?
In the very unlikely case that something goes wrong during the study it is important to understand that the university has procedures in place for reporting, investigating, recording and handling adverse events. Any complaints will be taken seriously and should be made to the appropriate authority. This might be the Chief Investigator and/or the University, depending upon who is involved in the research. The University will provide an indemnity statement for research that has been approved through the appropriate governance and ethical review processes.

5. Will My Taking Part be Confidential?
All data will be held securely and in confidence and that any identifiers will be removed prior to publication as required under Data Protection (1998) legislation.

Participants will be given a randomised subject number in order to keep anonymity, so data cannot be traced or used to identify the participant. Data will to be stored on file for 10 years in accordance with University Policy from the end of the data collection process. All electronic data will be put into a secure, password-locked electronic file and stored within Ulster University. Personal data and information (such as informed consent and health history questionnaires) will remain confidential unless agreed otherwise by an individual participant and will be kept on a password protected database or locked filing cabinet. All passwords and keys will be held by Josh Williamson. Only the chief investigator, Professor Gareth Davison, and Josh Williamson will have access to this data, before being adequately discarded. Names will not be linked to the information in accordance with the Data Protection Act (1998).

6. Do I Have to Take Part?

It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep. You will also be asked to sign a consent form. If you choose to take part, you can change your mind at any time and withdraw from the study without giving a reason.

7. What will happen my samples?

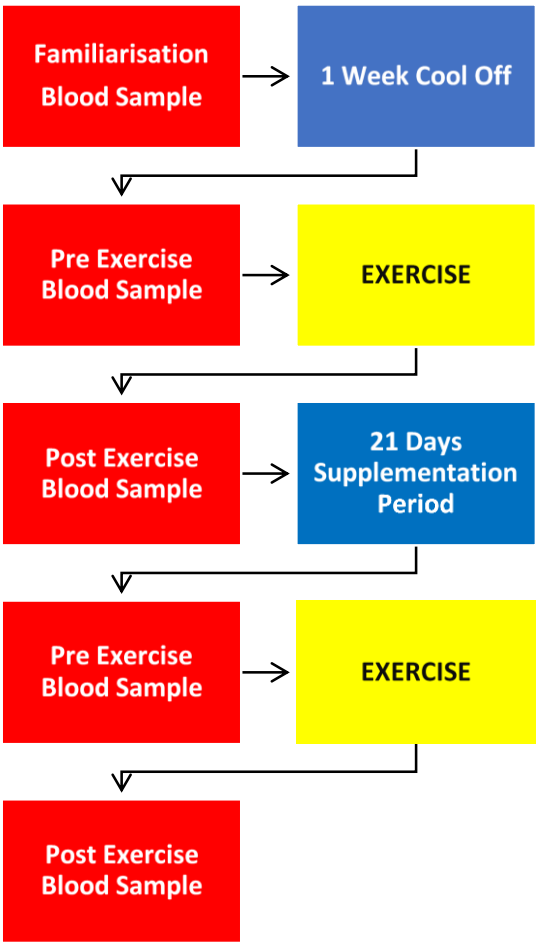
After blood and muscle samples are taken, they will be frozen and stored until they are ready to be analysed. Once the associated analysis has been performed, all samples will be destroyed via incineration. If you allow for enduring consent, samples will be retained for future research in the event of further questions and/or analysis in this area of research.

8. Contact Details

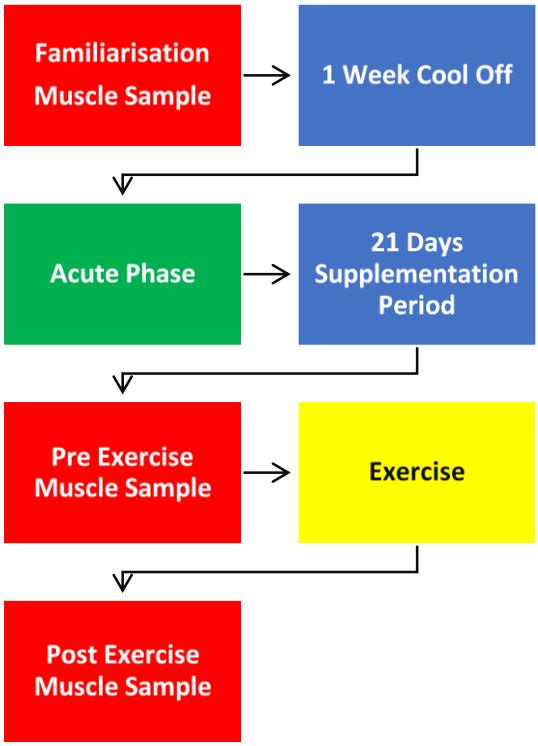
If you have any further questions or queries, feel free to contact me on the following information;

Josh Williamson – Williamson-J5@ulster.ac.uk
Gareth Davison – gw.davison@ulster.ac.uk

Venous Blood Collection



Muscle Tissue Collection



Participant Information Sheet

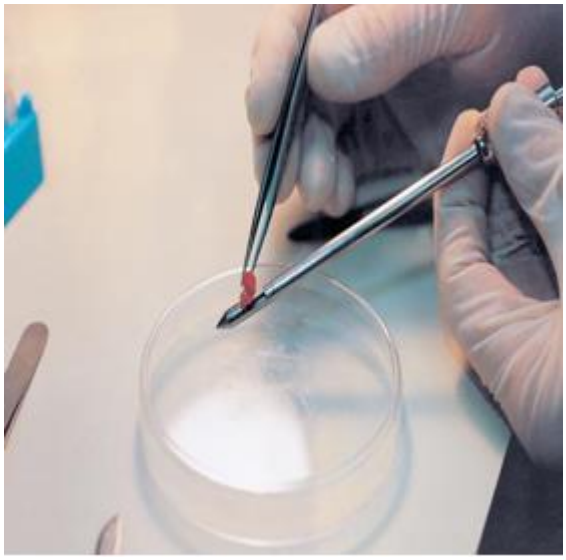
(Additional information for muscle biopsy procedure and care)

High Intensity Intermittent Exercise and Mitochondrial Targeted Antioxidant Supplementation; Effect on DNA and RNA Modification

This sheet will provide additional information to the information already provided in the participant information sheet. It contains an overview of what a muscle biopsy is, why they are being performed in this study and most importantly, what having a biopsy will involve for you as a participant. As with the participant information sheet, it is important that before you consent to partaking in this procedure you understand and are comfortable with what is being asked of you. You are under no obligation to partake, moreover if you do give your consent you are still free to withdraw, at any point, without explanation.

What is a muscle biopsy?

A muscle biopsy is a commonly performed procedure that is used in research studies and medical diagnoses. It involves the removal of a small (no more than 150mg) piece of muscle tissue from the leg as illustrated in the photo below. In this study, the sample will be taken from the large muscle found at the front of your thigh.



Biopsy Procedure. The muscle biopsy procedure involves a small muscle sample being extracted through the skin using a sterile, hollow needle within an automated biopsy device. This allows for the biopsy to be performed with little or no discomfort.

To begin, the skin covering your thigh muscle will be carefully cleaned. A small amount of local anaesthetic (to numb the area) will be injected into and under the skin. You may experience a tingling sensation while the anaesthetic is injected. Once the area is completely numb, a small, 0.4 – 0.5 cm incision will be made in your skin in order to create an opening for the biopsy needle. You should not feel this due to the anaesthetic.

The biopsy device will then be inserted through the incision into the leg muscle which again you should be unable to feel. You will hear a clicking noise as the biopsy device is activated and you may feel a strange pulling sensation that may cause a few seconds of discomfort, this will pass very quickly. Pressure will be applied to the site to minimise any swelling or bruising deep within the muscle, this helps with the healing process. This will also stop any bleeding that may occur.

When the wound is dry, sterile strips (steri-strips) will be applied to close the incision and the area will be covered with a sterile dressing. This is to ensure that the area remains clean and to minimise the risk of infection. In the 24-48 hours after the biopsy, it is important that you keep the sterile dressing in place and dry. Therefore, it is advised that where possible you don't shower or if you do, that you apply a water tight covering to the area. If the dressing does come loose or become wet, contact the research team who can arrange to have the area re-dressed.

Aftercare. Immediately after the biopsy you should feel no discomfort. As part of the research protocol you will be asked to exercise soon after the biopsy and again you should feel no discomfort during this period. You will be under close supervision from the research team at all times and their primary role is to ensure your safety and comfort. Following experimental exercise, you may have a second biopsy taken from the same site using the same protocol as outlined above.

After experimental exercise, you should refrain from excessive muscle (e.g. physical activity, exercise and manual labour) use for the remainder of the day. Once the anaesthetic wears off, your leg may feel a bit tight and some people have the sensation of a deep bruise. If you do experience discomfort associated with the biopsy, you may wish to use basic pain killers such as Paracetamol. It is also beneficial to periodically apply an ice pack to the biopsy site the following day, as this will help to reduce any swelling and any residual soreness. The following day your leg may feel tight and you may feel a slight discomfort when going down stairs, similar to the discomfort associated with post exercise muscle soreness experienced after a hard exercise. You should be able to comfortably exercise at normal capacity within 2 days of the biopsy.

Seven to 10 days after the biopsy you will be contacted by the research team to enquire as to how the biopsy site is healing and answer any questions you may have. You will be provided with contact details for the research team should you wish to discuss anything further in the interim.

The picture below demonstrates what is to be expected in the weeks following the muscle biopsy. It should also be noted, these will continue to heal over time.

Appendix D

Sport and Exercise Science Research Institute

Faculty of Life and Health Science

Consent Form

The Effects of Aerobic Exercise and Barley Grass Supplementation on DNA Damage

	Please Initial
• I confirm that I have been given and have read and understood the information sheet for the above study and have asked and received answers to any questions raised	[ ]
• I understand the nature and risks associated with blood draws. I confirm and consent for blood collection and storage of the stated amounts.	[ ]
• I understand the nature of the study and understand the possible side effects of barley grass consumption i.e. allergic reaction, gastrointestinal disturbances, nausea etc.	[ ]
• I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason and without my rights being affected in any way	[ ]
• I understand that the researchers will hold all information and data collected securely and in confidence and that all efforts will be made to ensure that I cannot be identified as a participant in the study (except as might be required by law) and I give permission for the researchers to hold relevant personal data	[ ]
• I agree to take part in the above study	[ ]

I (name)

.....

of (address)

.....

.....

hereby consent to take part in the above investigation, the nature and purpose of which have been explained to me. Any questions I wished to ask have been answered to my satisfaction. I understand that I may withdraw from the investigation at any stage without being required to give a reason for doing so.

Signed (Volunteer)

..... Date .....

(Investigator)

..... Date .....

Sport and Exercise Science Research Institute

Faculty of Life and Health Science

Consent Form

Effects of Exercise in Hypoxia on DNA Oxidation, Repair and Gene Expression

	Please Initial
• I confirm that I have been given and have read and understood the information sheet for the above study and have asked and received answers to any questions raised	[ ]
• I understand the nature and risks associated with blood draws. I confirm and consent for blood collection and storage of the stated amounts.	[ ]
• I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason and without my rights being affected in any way	[ ]
• I understand that the researchers will hold all information and data collected securely and in confidence and that all efforts will be made to ensure that I cannot be identified as a participant in the study (except as might be required by law) and I give permission for the researchers to hold relevant personal data	[ ]
• I agree to take part in the above study	[ ]

I (name)

.....

of (address)

.....

hereby consent to take part in the above investigation, the nature and purpose of which have been explained to me. Any questions I wished to ask have been answered to my satisfaction. I understand that I may withdraw from the investigation at any stage without being required to give a reason for doing so.

Signed (Volunteer)

..... Date .....

(Investigator)

..... Date .....

Sport and Exercise Science Research Institute
Faculty of Life and Health Science
Consent Form

High Intensity Intermittent Exercise and Mitochondrial Targeted Antioxidant Supplementation; Effect on DNA and RNA Modification

Please confirm, by marking the boxes, that you agree with the following statements:

1. I have been given and have read and understood the information sheet for the above study and have asked and received answers to any questions raised ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason and without my rights being affected in any way ☐

3. I understand that the researchers will hold all information and data collected during the study securely and in confidence and that all efforts will be made to ensure that I cannot be identified as a participant in the study (except as might be required by law) and I give permission for the researchers to hold relevant personal data ☐

4. I understand that my blood or other tissues are required for the purposes of this study and confirm that I have been given details of the amount(s) to be taken and how it will be stored, used and the method of disposal ☐

5. I agree to take part in the above study ☐

6. The potential benefits of keeping my blood or other tissues for future research studies have been explained to me and (please read carefully and choose ***ONE***):

   a. I consent to their indefinite storage and use in any University-approved future study, ☐

   OR

   b. I consent to their indefinite storage and use in any University-approved future study that does not involve the use of my genetic material; ☐

   OR

   c. I do not wish my blood or tissues to be used for any purpose other than this study ☐

Name of Participant (please print)	Signature	Date (dd/mm/yy
Name of Researcher	Signature	Date (dd/mm/yy)

Appendix E

HEALTH HISTORY QUESTIONNAIRE

Any information contained herein will be treated as confidential

Please answer all questions. Circle appropriate answer

Name:_____ Date of Birth:_____/_____/_____

Medical History

Personal History

1.

Have you ever fainted or passed out when exercising?	Yes	No
Do you ever have chest tightness?	Yes	No
Does running ever cause chest tightness?	Yes	No
Have you ever had chest tightness, cough or wheezing that made it difficult for you to perform in sports?	Yes	No
Do you have trouble breathing or do you cough during or after activity?	Yes	No
Have you ever been dizzy during or after exercise?	Yes	No
Have you ever had chest pain during or after exercise?	Yes	No
Do you have or have you ever had racing of you heart or skipped heartbeats?	Yes	No
Have you ever been told you have a heart murmur?	Yes	No
Do you get tired more quickly than your friends do during exercise?	Yes	No
Have you ever been told you have a heart arrhythmia?	Yes	No
Do you have any other history of heart problems?	Yes	No
If you have answered YES to any of the above please give details:		

2.

Have you ever had a seizure?	Yes	No
Have you ever been told:-		
You have epilepsy?	Yes	No
To give up sports because of health problems?	Yes	No
You have high blood pressure?	Yes	No
You have high cholesterol?	Yes	No
You have had rheumatic fever?	Yes	No
You have lung disease?	Yes	No

You have diabetes?	Yes	No
You have thyroid disease?	Yes	No
Have you ever been treated/hospitalised for asthma?	Yes	No
Do you have any allergies?	Yes	No
Have you had a severe viral infection (e.g. myocarditis or mononucleosis) within the last month?	Yes	No
Are you taking any medication at the present time?	Yes	No
Have you routinely taken any medication in the past two years?	Yes	No
If you have answered YES to any of the above please give details:		

3.

Have you had to consult with your doctor within the last six months?	Yes	No
If YES, please give details of reasons, which may affect your participation in the test(s)		

4.

Do you currently have any form of muscle or joint injury?	Yes	No
If YES, please give details which may affect your participation in the test(s):		

5.

Have you had any reason to suspend your normal activity in the past two weeks?	Yes	No
If YES, please give details which may affect your participation in the test(s):		

6.

Is there anything to your knowledge that may prevent you from successfully completing the task(s) that have been explained to you?	Yes	No
If YES, please give details which may affect your participation in the test(s):		

Signature of Participant_____Date_____/_____/_____

Signature of Test Supervisor_____Date_____/_____/_____

Please supply the name, address and telephone number for an emergency contact